



The role of genetic variation in VKORC1 and GGCX on warfarin response in a South African population

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Abstract

Background: Warfarin is the most commonly used drug in the management of thromboembolic disease. However, there is a huge variability in the management of warfarin affecting the international normalized ratio (INR). Many genetic-association studies have reported on European and Asian populations which has led to the designing of specific algorithms that are now being used to assist in warfarin dosing. However, very few or no studies have looked at the pharmacogenetics of warfarin in African populations, yet, huge differences in dosage requirements to reach the same INR have been observed. We set out to investigate genetic factors affecting warfarin therapy in South African black (SAB) and Mixed Ancestry (MA) participants.

Methods: We recruited consenting participants from Groote Schuur Hospital and Gugulethu Community Health Centre comprising 263 of Mixed ancestry (MA) descent and 109 of South African black (SAB) descent. Demographic data, clinical information and 5ml of blood for genetic characterization was obtained from participants. DNA was extracted from the blood and genotyped using PCR/RFLP and Sanger sequencing for 5 single nucleotide polymorphisms (SNPs), *VKORC1* c.-1639G>A, *VKORC1* c.1173C>T, *VKORC1* c.3730G>A, *GGCX* c.1218C>T and *GGCX* c.1242C>T.

Results: We report an association of age with warfarin dose, where, older patients had lower dose requirements ($p > 0.001$ in MA; $p > 0.042$ in SAB). Higher BMI was associated with increased stabilizing dose in the MA group ($p = 0.003$). The SNP *VKORC1* c.-1639G>A was associated with stabilizing dose only in MA ($p > 0.003$), with carriers of the A/A genotype requiring lower dosages (18 ± 14 mg/week) compared to G/G (38 ± 14 mg/week).

Conclusion: We conclude that environmental and genetic factors may play a role in the way South African patients respond to warfarin. Particularly, it appears as if MA and SAB population group have different underlying genetic markers for warfarin response. This knowledge in warfarin pharmacogenomics could help in the judicious use of warfarin in South African patients. However, more genetic markers need to be characterized to understand the profiles of genetic determinants affecting responses to warfarin therapy in world populations.

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List of Abbreviations

ADR	Adverse drug reactions
AKI	Acute kidney injury
ACC	African Anticoagulation clinics
AF	Atrial fibrillation
ANOVA	Analysis of variance
APOE	Apolipoprotein
BMI	Body mass index
bp	Base pair
Calu	Calumenin
CKD	Chronic kidney disease
COAG	Clarification of Optimal Anticoagulation
CYP	Cytochrome P450 isoenzyme
DVT	Deep vein thrombosis
dNTP	Dinucleotide triphosphate
DNA	Deoxyribonucleic acid
DOAC	Direct oral anticoagulant
EDTA	Ethylenediaminetetraacetic
ETOH	Ethanol
EU-PACT	European Pharmacogenetics of Anticoagulant Therapy
FDA	Food and Drug Administration
FWS	Fetal warfarin syndrome
G	Grams
GCHC	Gugulethu Community Health Centre
GGCX	Gamma-glutamyl carboxylase
GSH	Groote Schuur Hospital
HREC	Human Research Ethics Committee
HWE	Hardy-Weinberg equilibrium
INR	International normalised ratio
ISI	International Sensitivity Index
mg	Milligrams
MgCl ₂	Magnesium Chloride
ml	Millilitres

mM	Millimolar
NaCl	Sodium Chloride
NaOAc	Sodium Acetate
NOACs	New oral anticoagulants
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
PE	Pulmonary embolism
PK	Pharmacokinetics
RCT	Randomized Control Trial
RE-LY	Randomized Evaluation of Long-Term Anticoagulation Therapy
RFLP	Restriction fragment length polymorphism
SA	South Africa
SDS	Sodium dodecyl sulphate
SSA	Sub-Saharan Africa
SNP	Single nucleotide polymorphism
TBE	Tris Borate EDTA Buffer
TE	Tris EDTA
TF	Tissue factor
TWD	Total weekly dose
U	Units
μl	Micro litre
μM	Micro molar
USA	United State of America
UTR	Untranslated region
VKA	Vitamin k antagonist
VKDCF	Vitamin K Dependent Clotting Factors
VKORC1	Vitamin K epoxide reductase complex subunit 1
V	Volts
Vs	Versus
VT	Venous thromboembolism

w/v

Weight by volume

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Chapter 1: Introduction and literature review

1.1 Pharmacogenomics/Pharmacogenetics

To assess drug efficacy, dose and safety; it is important to consider inter-individual variations in pharmacokinetics (drug absorption, distribution, metabolism and excretion) and pharmacodynamics (drug targets) (Pirmohamed, 2013). Variations in drug response such as adverse drug reactions have led to differences in the efficacy of drug treatments among individuals (Kudzi *et al.*, 2011). Pharmacogenetics and pharmacogenomics, makes it possible to identify individuals likely to benefit or not from a particular treatment and those who may experience life threatening adverse reactions from a particular drug (Giacomini *et al.*, 2007). Pharmacogenomics incorporated into drug discovery enables the identification of new drug targets, reduction in treatment costs, improvement in understanding on the mechanism of action of drugs to improve efficacy and reduce adverse drug reactions (ADRs) (Kudzi *et al.*, 2015). In addition, pharmacogenetics may assist in improving the ability of a country to respond to population specific disease threats and enable improved policy decisions on interventions for population specific healthcare system (Pang *et al.*, 2009). Unfortunately for African populations there is limited information available on the pharmacogenetics of indigenous African populations (Marsh, 2008) making ADRs inevitable and more common among the African population (Arwood *et al.*, 2017). In addition, more clinical trials are carried in populations that do not include Africans, thus, in most cases ADRs are encountered post marketing when the drug has made it into Africa for use. For example, the use of efavirenz in sub-Saharan Africa has resulted in frequent observations of ADRs (Masimirembwa *et al.*, 2016).

1.1.1 Adverse drug reactions

Adverse drug reactions (ADRs) are complications caused by drug treatment which may result in morbidity, mortality and hospitalization in patients, more especially in patients living in developing countries like South Africa where not only is the patient affected but also the healthcare system is affected severely by ADRs (Dandara *et al.*, 2011; Lövborg *et al.*, 2012; Bracken *et al.*, 2017). A systematic review by Smyth *et al.* (2012) reported that ADRs cause hospitalization in children in incidences ranging from 0.6-16.8% (Smyth *et al.*, 2012). This rate is similar to adult hospitalization rate reported by Davies *et al.* (2009) of 14.7% in Liverpool

(Davies *et al.*, 2009). The incidence rates suggest the importance of studying how to avoid (prevent) ADRs (Ferner and Aronson, 2010). In sub-Saharan Africa, Masimirembwa *et al.* (2016) reported on the ADRs caused by efavirenz causing neuropsychiatric conditions (Masimirembwa *et al.*, 2016). It is important to note that hospitalisation due to ADRs is an additional burden that consumes scarce resources in poor and developing countries. According to World Health Organisation (WHO) (2009), in developing countries, one out of ten patients is harmed during hospital treatment caused by unsafe medical care (WHO, 2009, 2011).

In a meta-analysis conducted by Hakkarainen *et al.* (2012) it was concluded that healthcare systems are significantly affected by avoidable ADRs which result in morbidity among patients (Hakkarainen *et al.*, 2012). They further estimated that half of all ADRs may be avoidable in 52% of outpatients and 45% of adult patients (Hakkarainen *et al.*, 2012). Similar findings were reported by Mouton *et al.* (2016) in a South African study that 45% of ADRs that led to administration could be prevented (Mouton *et al.*, 2016). However, the lack of commonly accepted terms/definitions has made the study of how to avoid ADRs complex (Ferner and Aronson, 2010). This study is focussing on the use of warfarin, a vitamin K antagonist, and factors affecting warfarin therapy.

1.2 Vitamin K antagonists

Vitamin k antagonists (VKAs) have been the primary oral anticoagulant drugs for the past decades in the treatment and prevention of arterial and venous thromboembolic events (Ageno *et al.*, 2012; Caudle *et al.*, 2016). The understanding of the pharmacokinetics and pharmacodynamics of VKAs has been attributed to laboratory and clinical studies bringing to light interactions of VKAs, antithrombotic effects and the risks of using VKAs (Ageno *et al.*, 2012). The basis of VKAs mechanisms of action is that they exert their thrombotic effect through interference with cyclic conversion of vitamin K and vitamin K epoxide (2, 3 epoxide) (Figure 1.1). As the VKAs have their antithrombotic effect, the gamma-carboxylation of the glutamate residues follows (Stafford, 2005; Ansell *et al.*, 2008). In turn, gamma-carboxylation is needed by the vitamin k-dependent coagulation factors (II, VII, IX and X) to activate their coagulant activity (Figure 1.1) (Ansell *et al.*, 2008).

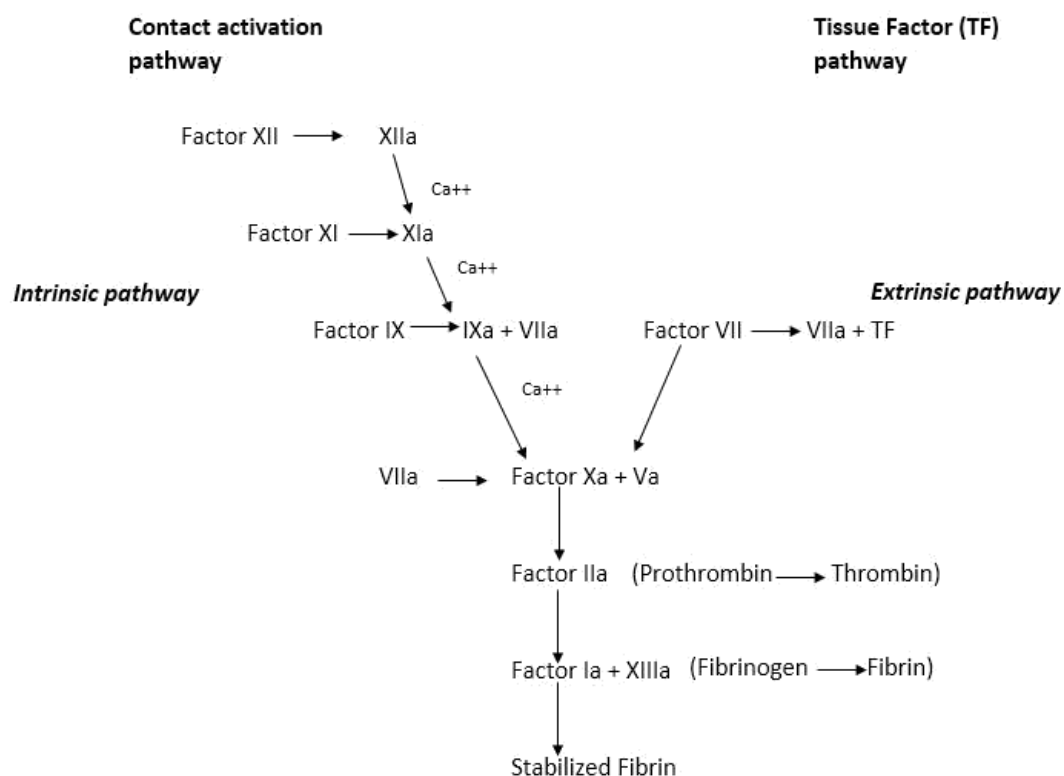


Figure 1.1: Coagulation cascade- The intrinsic and extrinsic pathway. The pathway of the activation of coagulation factors, adapted from (Davie, Fujikawa and Kiesel, 1991).

Vitamin k antagonist treatment promotes the production of hepatic proteins that are partially carboxylated and decarboxylated with low coagulant activity (Friedman *et al.*, 1977; Malhorta *et al.*, 1985). A calcium-dependent conformational change in coagulation proteins requires carboxylation which leads to the binding of cofactors to phospholipid surfaces (Borowski *et al.*, 1986; Ageno *et al.*, 2012). For carboxylation to occur, the reduced form of vitamin k, gamma-carboxylase, carbon dioxide and molecular oxygen are required (Whitlon *et al.*, 1978). An example of a vitamin k antagonist is warfarin. In addition to warfarin, there are several other anticoagulants including the new oral anticoagulants (NOACs) such as apixaban, rivaroxaban, dabigatran and edoxaban, however, our focus is on warfarin as it remains the most used anticoagulant world-wide (Fohner *et al.*, 2015).

1.2.1 New oral anticoagulants

Warfarin is a primary vitamin k antagonist however; in recent years, four new oral anticoagulants (NOACs) have been introduced, targeting factors of the clotting cascade dabigatran, apixaban, rivaroxaban and edoxaban (Table 1.1) (Barnes *et al.*, 2015). Favourable safety profiles have been

observed in the use of NOACs. Though expensive, NOACs have been favoured by both patients and providers because they are easy to use and efficient (Barnes *et al.*, 2015). It is for these reasons that they are used for stroke prophylaxis caused by atrial fibrillation (AF) and for treating and preventing venous thromboembolism (VT). However, there are still concerns about underutilization of NOACs (Ogilvie *et al.*, 2010). With an estimated 3 million patients with AF and VT of whom 75 000 patients out of the 3 million patients are diagnosed annually. This indicates that the underutilization of NOACs may have clinical implications (Mozaffarian *et al.*, 2015). Anticoagulant therapy for AF patients, to date, has not shown any improvements in underutilization of oral anticoagulants and this was observed through a nationwide analysis (Kirley *et al.*, 2012; Mahan *et al.*, 2012; Chugh *et al.*, 2014; Patel *et al.*, 2014).

Table 1.1: Novel oral anticoagulants

Drug	Brand name	Target	Trial	Reference
Dabigatran	Pradaxa®	Factor IIa	RE-LY	(Connolly <i>et al.</i> , 2009)
Apixaban	Eliquis®	Factor Xa	ARISTOTLE	(Granger <i>et al.</i> , 2011)
Rivaroxaban	Xarel®	Factor Xa	ROCKET AF	(Patel <i>et al.</i> , 2011)
Edoxaban	Lixiaa®	Factor Xa	ENGAGE AF-TIMI 48	(Giugliano <i>et al.</i> , 2013)

A process called contact activation activates factor XII to begin the process of coagulation (Renné and Gailani, 2007). Following the activation of factor XII, sequential activation of factor XI and IX proceed (Gailani and Renné, 2007). In the extrinsic pathway, activated plasma factor VII combines with tissue factor to initiate coagulation through injury (Mackman, 2004; Morrissey, 2004). It is at level Xa where the intrinsic and extrinsic pathway unit activating thrombin to fibrin to complete coagulation.

1.2.2 Warfarin use as an oral anticoagulant

Warfarin, a vitamin k antagonist, was introduced in the 1950s and it has since become the most commonly used oral anticoagulant worldwide (Table 1.2) (Hirsh, 1992; Hirsh *et al.*, 2001; Kornfield *et al.*, 2012; Cho *et al.*, 2016). It is a relatively inexpensive drug, however, through cost-analysis it has shown that the basis of warfarin dosage being monitored frequently makes the therapy more expensive (Anderson *et al.*, 2002; Botteman *et al.*, 2002; Stambler and Ngunga, 2015). For warfarin therapy, the primary aim of is to maintain a stable International normalised ratio (INR) with the lowest effective dose possible (Kuruvilla and Gurk-Turner, 2001; Pirmohamed, 2013). In healthy people the ideal INR range is between 0.8-1.1. In general,

among patients on warfarin, the INR is expected between 2.0 to 3.5 (Hirsh *et al.*, 2001; Kearon *et al.*, 2008; Sonuga *et al.*, 2016). The INR is a standardized measurement used to measure prothrombin (time taken for blood to clot) and serves as a crucial aspect of warfarin-related genetic studies. In addition, it enables the exchange and comparison of information between laboratories as well as different types of studies (Kamali and Wynne, 2010; Wells *et al.*, 2014). The understanding of how INR works is important as it shows the correlation of variants with their phenotype with which genes (Eriksson and Wadelius, 2012; Lam and Cheung, 2012). International efforts have shown that the contribution of different variables include genetic variants among populations may cause trans-racial differences in warfarin dose requirements (Limdi *et al.*, 2010; Wypasek *et al.*, 2016). An international standard is used to test thromboplastin and this relationship is expressed as the International Sensitivity Index (ISI). INR is calculated as follows:

$$INR = (patient\ prothrombin\ time / mean\ normal\ prothrombin\ time) ISI$$

Table 1.2 Indicates warfarin prescriptions for different populations

Population	Year	Warfarin prescriptions	Reference article
US	2010	>30 million	(Kirley <i>et al.</i> , 2012)
Canada	2013	5 million	(Weitz <i>et al.</i> , 2015)
Ireland	2010	57 000	(Health Service Executive, 2014)
South Africa	2012	427 238	(Blaauw, 2012)

Warfarin is used for the prevention and treatment of thromboembolic complications linked with AF and thrombosis (Kuruvilla and Gurk-Turner, 2001; Kornfield *et al.*, 2012). Treatment with warfarin is notably complicated and time consuming as it requires that an adequate dose is established for each individual specifically, and this is achieved by monitoring the anti-coagulant status using INR. Inter-individuality among warfarin patients is influenced by many factors including genetics (variations of the gene encoding the enzymes linked with the pharmacokinetics and pharmacodynamics of warfarin) and the non-genetic factors include race, age, height, body weight and diet (Rieder *et al.*, 2005; Aquilante *et al.*, 2006; Gage *et al.*, 2008; International Warfarin Pharmacogenetics Consortium *et al.*, 2009; Takeuchi *et al.*, 2009; Yoshizawa *et al.*, 2009; Johnson and Cavallari, 2013). Dosing algorithms have been developed because of the observation that genetic and non-genetic variables account for nearly 50% of the variance in warfarin dose (International Warfarin Pharmacogenetics Consortium *et al.*, 2009; Johnson *et al.*, 2011). Furthermore, proteins C and S which are naturally occurring endogenous anti-coagulant as well as clotting factors II, VII, IX and X are inhibited by warfarin (Horton and Bushwick, 1999; Njovane *et al.*, 2013).

The anticoagulant effect of warfarin has high implications on adverse drug reactions causing hospital admissions (Pirmohamed, 2013). One of those reactions include bleeding, with the risk being directly proportional with the intensity of anticoagulation as indicated by INR value (Hylek *et al.*, 2007; Li *et al.*, 2015). For instance, Pirmohamed *et al.* (2013) reported that an elderly population with an INR value >4.1 has a 25% risk of bleeding compared with those presenting with an INR between 2.0-3.0 (Pirmohamed *et al.*, 2013). Consequently, administering fresh frozen plasma can reverse warfarin effect therefore limiting adverse reactions (Hanley, 2004).

Adverse reactions such as haemorrhage in a US study by Hylek *et al.* (2007) have raised safety concerns about warfarin which has led to the discontinuation of the drug in the first year in about 26% of the elderly population (Hylek *et al.*, 2007). It is for this reason that positive approaches have been implemented to improve anticoagulation control in warfarin patients and these include development of specialised anticoagulation clinics, initiating computerised dosing software programmes as well as dosing algorithms which help in obtaining accurate dosing. In addition, development of educational programmes for the patients (Rose, 2012) has had a huge positive effect. It has been found that patients appropriately trained in using a hand-held INR monitoring device to be able to monitor INR independently (Pirmohamed *et al.*, 2013). Unfortunately, these measures have not been fully implemented in clinical practice (Pirmohamed

et al., 2013). However, patient self-management may be less expensive than anticoagulation clinic visits (Connock *et al.*, 2007).

1.2.2.1 Warfarin resistance and warfarin sensitivity

The inability to prolong the prothrombin time or raise INR to the optimal range when warfarin is administered at a normally prescribed dose is known as warfarin resistance (Lakshmi *et al.*, 2012). A higher warfarin dose does not necessarily provide a diagnosis for warfarin resistance, in addition it is difficult to determine variation by patient population (Lakshmi *et al.*, 2012). This difficulty is caused by dietary factors and establishing normal metabolic variations for individuals. A warfarin resistant patient is one that requires a dose of more than 105 mg per week which is equivalent to 15 mg/day. To reverse the effects of warfarin, warfarin resistant patients require lower doses of vitamin k (Thijssen, 1987; Hulse, 1996; Linder, 2001; Ansell *et al.*, 2004). Warfarin resistance can be acquired or hereditary (Hulse, 1996; Vaes and Chyka, 2000). Acquired warfarin resistance is caused by difficulties in complying to warfarin therapy, increased consumption of vitamin k (Lakshmi *et al.*, 2012), warfarin absorption decreased (Daly and Aithal, 2003), rapid warfarin clearance (Cain *et al.*, 1997) and drug interactions (Vaes and Chyka, 2000; Wong and Chan, 2003). Hereditary warfarin resistance is thought to be caused by genetic factors that may either lead to enhanced metabolism of the drug (Pharmacokinetics) or reduced drug activity (Pharmacodynamics) (Lakshmi *et al.*, 2012). Polymorphisms also have an influence, variant alleles in *VKORC1* and *CYP2C9* are associated with increased warfarin sensitivity (Lakshmi *et al.*, 2012).

1.2.2.2 Pharmacokinetics and pharmacodynamics of warfarin

Warfarin interferes with vitamin k recycling in the liver, this affects vitamin k epoxide reductase complex (*VKORC1*) the main protein in vitamin k recycling (Li *et al.*, 2004; Rost *et al.*, 2004). This interference causes inactive vitamin k-dependent proteins to be secreted (Dahlbäck, 2005). Calumenin (*CALU*) inhibits the effects of warfarin by binding to vitamin k reductase (Wajih *et al.*, 2004). Other genes involved in vitamin k recycling is apolipoprotein E (*APOE*) and gamma glutamyl carboxylase (*GGCX*).

Warfarin occurs as a stereoisomer of R- and S-enantiomers. The potency of S-warfarin is 3-5

times more than the R-warfarin (Jaffer and Bragg, 2003; Ageno *et al.*, 2012). Warfarin is a water-soluble drug that has good bioavailability. It is absorbed from the gastrointestinal tract and it is able to reach maximum blood concentrations within 90 minutes after administration (Kelly and O'Malley, 1979). The half-life of racemic warfarin is 36 to 42 hours (S-enantiomer 29 hours, R-warfarin 45 hours) (Jaffer and Bragg, 2003). Racemic warfarin is protein bound and accumulates in the liver where the S and R-warfarin are transformed metabolically by different pathways (Jaffer and Bragg, 2003).

The S-warfarin is primarily metabolised by the cytochrome P450 2C9 enzyme which catalyses the reactions of 6- and 7-hydroxylation (Kunze and Trager, 1996), the predominate metabolite being (S)-7-hydroxywarfarin (Rettie *et al.*, 1989) and minimally metabolised by CYP3A4 enzyme. In contrast, clearance of the R-warfarin forms 6-, 7-, 8- and 10-hydroxylated metabolites as well as alcohol reduction. Cytochrome P450 1A2 catalyses 6-hydroxylation, cytochrome P450 2C19 catalyses 8-hydroxylation and cytochrome P450 3A4 catalysis 10-hydroxylation (Figure 1) (Kunze and Trager, 1996). Other genes involved in R-warfarin metabolism include CYP1A1, CYP2C8, CYP2C18, CYP2C19 and CYP3A5 (Rettie *et al.*, 1992; Zhang *et al.*, 1995; Kaminsky and Zhang, 1997; Huang *et al.*, 2004). For this study, we focussed on two genes namely; VKORC1 and GGCX.

1.2.2.3 Common indications for warfarin anticoagulant use in Africa

Of all anticoagulants, warfarin remains the most effective for the treatment and prevention of a variety of prothrombotic cardiovascular, stroke and hematologic conditions (Petersen *et al.*, 1989; Hart *et al.*, 2007) and it is for this reason that it has been used for over 60 years (Avorn, 2011; Friberg *et al.*, 2012). Warfarin usage is commonly for the treatment and/or prevention of atrial fibrillation, pulmonary embolism, mechanical valve replacement and deep vein thrombosis (Lee and Klein, 2013).

Atrial fibrillation, a common cardiac arrhythmia, cases in the developed world has a high expected prevalence than in Africa because of the increased aging population observed in this region (Fuster *et al.*, 2011). Furthermore, Africa has a high incidence of under treatment of streptococcal infections which contribute to the increased burden of valvular AF in relation to rheumatic disease (Damasceno *et al.*, 2012; Bloomfield *et al.*, 2013). The high incidence is thought to be attributed to limited skilled staff and minimum public awareness about diagnosis, poor access to primary healthcare and quick treatment of suspected streptococcal infections. In a country like South Africa, a primary public health prevention strategy should minimise costly

diagnostic testing, be cautious of missed diagnosis and reduce unnecessary antibiotic treatment (Kromberg and Krause, 2013). Between 1990 and 2010, AF was the highest cardiovascular disease burden in sub-Saharan African (SSA) (Moran *et al.*, 2013). By 2050, the prevalence of AF is estimated to be higher in Africa than other continents (Rahman *et al.*, 2014). Furthermore, the prevalence of stroke as a result of AF is high and stroke resulting from AF is more disabling with a high mortality risk than stroke from other causes (Lin *et al.*, 1996; Camm *et al.*, 2010; Hannon *et al.*, 2010).

A hospital-based South African study reported that 4.6% of cardiology patients had AF, suggesting a 5.6% prevalence of cases per 100 000 populations per year (Sliwa *et al.*, 2010). In addition, there was a 5.5% prevalence of AF for patients admitted over a 10-year period in a cardiology institute in Ivory Coast (Coulibaly *et al.*, 2010). Similarly, Mbaye *et al.* (2010) on a Senegalese retrospective study, reported the prevalence of AF to be 5.4% with AF causing 66% cardiac disorders (Mbaye *et al.*, 2010). However, Shavadia *et al.* (2013) in Kenya, reported only 0.7% prevalence rate of AF (Shavadia *et al.*, 2013). Notably, the low rates of AF reported for African populations may not be a true reflection of the disease burden. These low rates may be as a result of care seeking behaviour and most importantly, the fact that access to health care facilities is a challenge in Africa (Stambler and Ngunga, 2015) and that most studies are only focussed on a few if not one hospital.

Globally, stroke is the second most common cause of death (Murray and Lopez, 1997; Stambler and Ngunga, 2015) with nearly 70% of these deaths occurring in developing countries especially in Africa (Connor *et al.*, 2005). Stroke was the leading cause of disability and death in 2010 within Africa (Moran *et al.*, 2013). Therefore, the knowledge of warfarin dose is important as it is used to prevent stroke (Connor *et al.*, 2005). It is estimated that more females die from stroke than males (Connor *et al.*, 2005). However, there is still a high rate of general anticoagulant morbidity and mortality. The knowledge of genetic and non-genetic variables is of interest because of the wide inter-individual differences and the low therapeutic index of warfarin that influences the dose requirements for warfarin (Cooper *et al.*, 2008; Takeuchi *et al.*, 2009; Kamali and Wynne, 2010; Skov *et al.*, 2012).

1.2.2.4 Burden of stroke and atrial fibrillation and use of warfarin

The prevalence of stroke was 1.63/1 000 and 1.14/1 000 in rural and urban Nigeria, respectively (Danesi *et al.*, 2007; Oldgren *et al.*, 2014). On the contrary, the prevalence of stroke in African

Americans was 3.8% in 2012 (Go *et al.*, 2014). In Ethiopia, a single-centred study reported the mean age of stroke patients was 53.2 years and mortality was 44.5% (Zenebe *et al.*, 2005). The annual incidence of stroke in South Africa is approximately 75 000 (Bertram *et al.*, 2012). Jowi and Mativo. (2008) reported on a Kenyan cohort that the mean age was 61 years and 40% of these patients had valvular heart disease with half also having not received any form of treatment (Jowi and Mativo, 2008). Treatment can be in the form of well managed anticoagulation control which enables warfarin to reduce the risk of stroke in AF patients through the maintenance of INR for at least 60-70% of the time (Connolly *et al.*, 2008; Morgan *et al.*, 2009). At the same time, warfarin therapy has a risk of haemorrhage and the effectiveness of warfarin can be reduced through the administration of vitamin k. Warfarin, however, has a longer mechanism of action than vitamin k thus re-administration of vitamin k is required for patients with ongoing bleeding complications (DeZee *et al.*, 2006). Complications in patient treatment results in significant costs for the patient (Bramkamp, 2006). Atrial fibrillation costs include anticoagulation cost, treatment of stroke associated with AF and INR monitoring (Stambler and Ngunga, 2015).

1.3 Inter-individual variability in response to warfarin

During the initiation phase, daily monitoring of INR is recommended because it is difficult to predict initial individual doses (Pirmohamed *et al.*, 2013). An INR range between 2.0-3.0 is recommended for most disease indications while cardiac valve procedures recommend a INR range between 2.0-3.5 (Hirsh *et al.*, 2001; Kearon *et al.*, 2008; Sonuga *et al.*, 2016). The maintenance of warfarin dose is essential. Warfarin has a narrow therapeutic range for INR therefore, sub therapeutic INR caused by under dosing results in thrombosis and supra therapeutic INR causes excessive haemorrhage (Natarajan *et al.*, 2013) leading to dose variability.

Warfarin response is associated with a high inter-individual dose variability to achieve INR, which is affected by demographic variables such as age, BMI, clinical factors and genetic variation in genes. The most commonly studied genes are *CYP2C9*, and vitamin k epoxide reductase complex subunit 1 coding gene, *VKORC1* (Caldwell *et al.*, 2007; Yang *et al.*, 2013). Other factors that cause INR fluctuations include concomitant illnesses, drug interactions, vitamin k intake, inaccurate dosing and laboratory results, and poor or non-compliance (Miao *et al.*, 2007; Hamadeh *et al.*, 2016).

1.3.1 Concomitant illness

The anticoagulant response of a patient is affected by various drugs and diseases (Demirkan *et al.*, 2000; Ageno *et al.*, 2012). Firstly, Howard-Thompson *et al.* (2014) recommend that patients with thyroid dysfunction be monitored carefully (Howard-Thompson *et al.*, 2014). They suspect that the catabolism of vitamin k-dependent clotting factors is decreased by hypothyroidism consequently decreasing the response to warfarin. They further observed a general trend of increase in warfarin dose with a decreasing INR (Howard-Thompson *et al.*, 2014). In contrast, the catabolism of vitamin k-dependent clotting factors is increased in hyperthyroidism with a general trend of decreased warfarin with an increased INR (Jaffer and Bragg, 2003).

Secondly, non-renal drug clearance and metabolism has generated increased attention on the potential effect on chronic kidney disease (CKD). These patients are at a risk of acquiring cardiovascular disease, bone disease and anaemia. Consequently, they require multiple drugs for treatments of these conditions (Anderson *et al.*, 2009; Knauf and Aronson, 2009; Weiner and Rifkin, 2009). Increased coagulation factors such as factor VIII, fibrogen and von Willebrand cause prothrombic events in CKD patients (Wattanakit and Cushman, 2009; Pavord and Myers, 2011; Lutz *et al.*, 2014). Expectedly, dose alterations are necessary for patients with CKD on drugs that primarily use renal excretion like warfarin (Limdi *et al.*, 2010).

Within warfarin initiation therapy, it is estimated that about 20.5% of patients experience a minimum of one episode of warfarin related nephropathy (Brodsky *et al.*, 2011; An *et al.*, 2013). Clinicians have acknowledged that it is more challenging to manage anticoagulation in patients with kidney impairment and that it is unfortunate that both kidney impairment patients as well as the general medical population receive similar initiation doses and are managed similarly (Elliott *et al.*, 2007; Genovesi *et al.*, 2008; Brodsky *et al.*, 2011; An *et al.*, 2013). It has been reported that assessing warfarin dose requirements is necessary for patients with CKD. These patients require lower warfarin maintenance doses, and if the disease is severe, anticoagulation control is impaired (Limdi *et al.*, 2009, 2010). Over-anticoagulation and under-anticoagulation are at an increased rate and major haemorrhage which is caused by over-anticoagulation is at a two-fold increased risk in CKD patients (Dreisbach *et al.*, 2003; Elliott *et al.*, 2007). These results were obtained from studies that assessed haemorrhage risk in dialysis patients undergoing warfarin therapy (Elliott *et al.*, 2007).

In a Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) trial, no significant differences were observed in acute kidney injury (AKI) between warfarin and dabigatran use (Shafi *et al.*, 2013; Escoli *et al.*, 2015). Interesting to note that in a post hoc analysis of RE-LY, AF dabigatran patients indicated a slower reduction in renal function than warfarin patients and the reduction of renal function was increased because of previous warfarin exposure and if the patient was diabetic (Böhm *et al.*, 2015). Nonetheless, it is still unclear about the impact of dabigatran and warfarin use in association with AKI in real-world clinical practice (Zhang *et al.*, 2016).

There is limited clinical knowledge on the effect of liver disease on warfarin therapy available. Clinicians do not know whether liver disease treatment together with warfarin treatment may have successful anticoagulation control or increase bleeding risk (Efird *et al.*, 2014). With the recent developments of direct oral anticoagulants, liver disease patients were excluded from the trials (Connolly *et al.*, 2009; Schulman *et al.*, 2009; Granger *et al.*, 2011; Patel *et al.*, 2011). As a result, there is currently no planned or existing trial that may assist with the management of liver disease patients requiring anticoagulation therapy (Efird *et al.*, 2014).

In anticoagulation, the imbalanced state of pro- and anticoagulants has been described as advanced liver disease and it is distinguished by hematologic abnormalities (Tripodi and Mannucci, 2011). Hindered clotting factor levels in synthetic liver damage may extend prothrombin time (Villa and Maria, 2012). In addition, patients with chronic liver disease are likely to have reduced serum albumin (liver protein), this is believed to be due to structural alterations in the molecule. Consequently, reducing binding ability and distribution in the body (Ivanov *et al.*, 2002). Furthermore, liver disease patients are at an increased risk of thromboembolism (Søgaard *et al.*, 2009; Efird *et al.*, 2014). The reduction of activity in protein C-induced inhibition is thought to affect the prothrombotic state (Tripodi and Mannucci, 2011).

The uncertainties on whether to seek anticoagulation therapy for liver disease patients still remains unresolved (Efird *et al.*, 2014). Patients with stable mild to moderate liver disease indicating anticoagulation are likely to be in a balanced state of anticoagulant factors and, therefore, the risk of anticoagulation is reduced. However, those with severe liver disease are poor candidates (Efird *et al.*, 2014). For these patients, it would be beneficial if clinicians

would consider the risks and benefits of using warfarin. Unfortunately, currently there are no models that can assist the clinicians with the identification of patients that can be on warfarin therapy without experiencing complications (Efird *et al.*, 2014) likely due to drug interactions.

1.4 Warfarin use and Drug interactions

Drug interactions are the most common cause of INR fluctuations which increase the risk of haemorrhage or coagulation. These interactions may be defined as pharmacokinetic (PK) or pharmacodynamic (PD) (Jaffer and Bragg, 2003). PK interactions affect drug absorption, protein binding and warfarin hepatic metabolism (for example cholestyramine affects warfarin absorption) (Jähnchen *et al.*, 1978; Jaffer and Bragg, 2003). In addition, Valproic acid and non-steroidal anti-inflammatory drugs (NSAIDs) change the binding of plasma protein to warfarin (Sands *et al.*, 2002). Some NSAIDs that have antiplatelet activity enhance the anticoagulation effect when administered together with warfarin (Knijff-Dutmer *et al.*, 2003). In contrast, PD interactions affect haemorrhage or coagulation through effects of *antiplatelet* or alterations in vitamin k catabolism (Jaffer and Bragg, 2003).

1.4.1 Vitamin k intake

A diet consisting of high levels of vitamin k consumption results in the increase of vitamin k clotting factors which consequently decrease the anticoagulant response to warfarin (Booth *et al.*, 1997). Thus, low vitamin k consumption leads to an increased anticoagulant response to warfarin (Jaffer and Bragg, 2003). It is therefore important to have a consistent vitamin k intake to afford accurate warfarin dosing (Darnell *et al.*, 2014). In a single serving, food that is highly concentrated with vitamin k is the green leafy vegetables such as turnip greens, spinach and broccoli (Booth and Centurelli, 1999). Unfortunately, patients are misinformed and have been advised to avoid such types of foods which are nutritious. Patients should be advised if they want to eat these types of foods to do so in moderation (Jaffer and Bragg, 2003). A healthy diet may include these types of foods (Jaffer and Bragg, 2003). However, environmental factors should not go unnoticed but be monitored on how they affect warfarin metabolism.

1.5 Environmental factors affecting warfarin metabolism

1.5.1 Alcohol

Alcohol use is among the strongest risk factors for major bleeding for individuals using warfarin (Efird *et al.*, 2013). For instance, warfarin patients are advised to use alcohol infrequently by American Heart Association, however, no specific evidence is cited (Hirsh *et al.*, 2003). For patients with genetic variants that affect the anticoagulation cascade and warfarin metabolism, alcohol use could have problematic implications for warfarin safety and consequently affecting dose requirements (Limdi *et al.*, 2008; McDonald *et al.*, 2009; Roth *et al.*, 2014). This particularly affects patients with genotypes that suggest lower warfarin dose requirements, to reach therapeutic anti-coagulation levels, to a higher risk of bleeding if the genetic status is not known (Roth *et al.*, 2015).

The exact mechanism is not clear in literature, this can include compromised hepatic metabolism and other patient-specific factors (Noureldin *et al.*, 2010). Roth *et al.* (2015) observed that patients with lower hepatic vitamin k reductase complex (VKORC) content as well as reduced warfarin clearance to be more likely to experience biochemical changes carried out by alcohol than patients who have a faster hepatic metabolism and do not have this genetic mutation (Roth *et al.*, 2015). Alcohol consumption increases warfarin metabolism, this reduces warfarin efficacy leading to an increased risk of clot formation. Contrary, acute alcohol consumption may also affect warfarin metabolism, this can increase the risk of haemorrhage (Fraser, 1997; Moore *et al.*, 2007).

1.5.2 Smoking

Components of tobacco smoke induce *CYP1A2* (R-warfarin is a substrate of *CYP1A2*) which is attributed to interaction of warfarin with smoking (Kroon, 2007). This interaction in humans causes increased risk of thrombotic events (Kroon, 2007; Angoulvant *et al.*, 2015). Warfarin clearance has been reported to be enhanced by smoking thereby suggesting the requirement of dose adjustments (Sohn *et al.*, 2015). On the contrary, no differences were observed in warfarin dose requirements for previous smokers or current smokers in a retrospective study (Whitley *et al.*, 2007). Angoulvant *et al.* (2015) reported that atrial fibrillation patients who smoke may be exposed to an increased risk of thromboembolic events and/or an increased risk of bleeding (Angoulvant *et al.*, 2015). However, there are minimum guidelines available on smoke

termination and the management of anticoagulation therapy (Reichert *et al.*, 2008; Nathisuwan *et al.*, 2011).

1.5.3 Pregnancy

Warfarin use is discouraged in pregnancy because it leads to foetal warfarin syndrome (FWS), a congenital abnormality that is caused by warfarin passing through the placenta because of its low molecular weight (Hall *et al.*, 1980; Yurdakök, 2012). Warfarin sensitivity in pregnancy is greatest between week 6 and 9 of gestation (Hall *et al.*, 1980; Bian *et al.*, 2012). Common features of FWS included skeletal abnormalities, nasal hypoplasia and stippled epiphyses (Kumar *et al.*, 2012). Furthermore, interference in the activity of the vitamin k reductase causes a depletion in the levels of vitamin k mineralization inhibitors osteocalcin and matrix gla protein in cartilages resulting in displaced calcium deposits in epiphysis. Foetal complications are thought to be associated with warfarin dose requirements (Basu *et al.*, 2016). Khamooshi *et al.* (2007) reported embryopathy and foetal loss occurring when warfarin administration is above 5 mg (Khamooshi *et al.*, 2007). The continuation of warfarin until late gestation can cause haemorrhages in foetal organs leading to mortality (Menger *et al.*, 1997; Hassouna and Allam, 2014). Other anomalies associated with FWS include microcephaly, short neck, upper airway obstruction, choanal atresia, growth retardation, laryngeal abnormalities, blindness, optical atrophy, deafness, telebrachydactyly, pectus carinatum, congenital heart defects, seizure, mental retardation, hydrocephalus, Dandy Walker malformation and agenesis of corpus callosum (Hall *et al.*, 1980; Hou, 2004; Mazibuko *et al.*, 2012).

1.6 Genetics of warfarin

Warfarin metabolism is affected by genetic polymorphisms in *CYP2C9* and *VKORC1* both of which account for 30%-40% of warfarin dose variation (Bodin *et al.*, 2005; Sconce *et al.*, 2005; Vecsler *et al.*, 2006). Genetic studies have identified genetic variants and other candidate genes which are associated with dose requirements. These genes include gamma-glutamyl carboxylase (*GGCX*), epoxide hydrolase 1 (*EPHX1*), calumenin (*CALU*) and *CYP2C19* (Wadelius *et al.*, 2007; Luxembourg *et al.*, 2010; Huang *et al.*, 2011). Figure 1.2 shows S and R- warfarin metabolism with some of the genes involved in the PK and PD of warfarin. For this study, we investigated polymorphisms in two genes: *VKORC1* and *GGCX* because of their role in the PK and PD of warfarin.

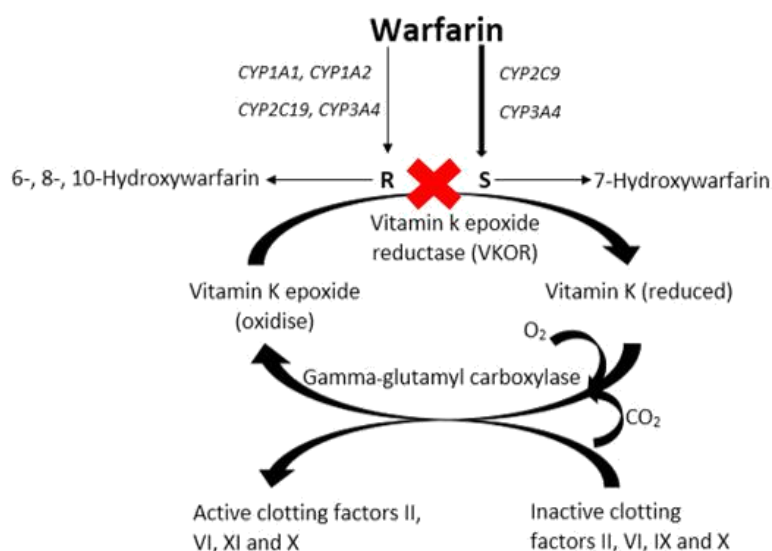


Figure 1.2: S- and R-warfarin metabolism in the vitamin k cycle. S-warfarin mainly metabolised by *CYP2C9* enzyme to 7-Hydroxywarfarin. R-warfarin mainly metabolised by *CYP1A2* and *CYP3A4* to 6-, 8-, 10- Hydroxywarfarin. Warfarin inhibits VKOR as indicated by the red cross therefore preventing the activation of clotting factors, adapted from (Tie *et al.*, 2011).

1.6.1 Cytochrome P450 2C9 (*CYP2C9*)

CYP2C9 is located on chromosome 10q24 (Ansell *et al.*, 2008) and is the main enzyme responsible for S-warfarin metabolism (Van Booven *et al.*, 2010) contributing 10% of dose variation in warfarin patients (Verhoef *et al.*, 2014). In addition, genetic variation in *CYP2C9* causes changes in enzyme activity leading to compromised metabolism (Van Booven *et al.*, 2010). Decreased *CYP2C9* due to polymorphisms results in increased concentrations of warfarin which are associated with increased bleeding. Literature has extensively investigated two non-synonymous single nucleotide polymorphisms (SNPs) within *CYP2C9* (*CYP2C9**2 - rs1799853 and *CYP2C9**3 - rs1057910) mostly among Caucasian and Asian populations (Yasar *et al.*, 1999; Yoon *et al.*, 2001; Burian *et al.*, 2002; Yildirim *et al.*, 2014). These SNPs have showed a strong association with warfarin response variability. Table 1.3 shows the comparative distribution of the two SNPs in different populations.

Table 1.3: Allele frequency for *CYP2C92 & *3 among different populations**

Geographical population	Racial population	<i>CYP2C9</i>*2	<i>CYP2C9</i>*3	Reference
Mozambican	African	0	0.01	(Vargens <i>et al.</i> , 2011)
South African	African	0	0.005	(Dandara <i>et al.</i> , 2011; Mitchell <i>et al.</i> , 2011)
Egyptian	African/Asian	0.117	0.092	(Shahin <i>et al.</i> , 2011)
Omani	Asian	0.074	0.029	(Tanira <i>et al.</i> , 2007)
Chinese	Asian	0.01	0.02	(Yuan <i>et al.</i> , 2005)
Turkish	Caucasian/Asian	0.13	0.1	(Ozgon <i>et al.</i> 2008)
African American	Caucasian	0.01	0.01	(Wu <i>et al.</i> , 2008)
British	Caucasian	0.14	0.085	(Sconce <i>et al.</i> 2005)

1.6.2 Vitamin k epoxide reductase complex 1 (*VKORC1*)

Warfarin resistance and clotting factor deficiencies are caused by variations found in the gene targeted by warfarin, *VKORC1* an enzyme located on chromosome 16, regulates the recycling of vitamin k 2,3 epoxide to vitamin k hydroquinone (Li *et al.*, 2004; Rost *et al.*, 2004; Natarajan *et al.*, 2013). With the inhibition of *VKORC1*, there is a blockage in the production of vitamin k which limits the availability of vitamin k epoxide in the liver consequently leading to pro-coagulation factors that are non-functional (Militaru *et al.*, 2015). Polymorphisms in *VKORC1* contribute 30% to warfarin dose requirements (Rost *et al.*, 2004). These include: -1639G>A (rs9923231), 1173C>T (rs9934438), 1542G>C (rs8050894), 2255C>T (rs2359612) and 3730G>A (rs7294) (Dean, 2012) (Table 1.4). There have been several studies that have examined the association of the -1639G>A (rs9923231) polymorphism found in the promotor region of *VKORC1* with warfarin dosing variability (Harrington *et al.*, 2004; Kimura *et al.*, 2007; Wu *et al.*, 2008; Sconce. *et al.*, 2009; Lenzini *et al.*, 2010; Natarajan *et al.*, 2013).

Table 1.4: Indicates allele frequency distribution of *VKORC1* SNPs among different populations

Population	c.-1639A	c.1173T	c.1542C	c.2255T	c.3730A	Reference
Mozambican	0.035	N/A	0.273	N/A	0.389	(Suarez-Kurtz <i>et al.</i> , 2010)
South African	N/A	0.04	N/A	0.24	0.40	(Dandara <i>et al.</i> , 2011)
Angolan	0.027	N/A	0.192	N/A	0.404	(Suarez-Kurtz <i>et al.</i> , 2010)
Brazilian	0.211	N/A	0.344	N/A	0.469	(Suarez-Kurtz <i>et al.</i> , 2010)
African American	0.110	0.120	0.300	0.210	0.440	(Perera <i>et al.</i> , 2011)

The *VKORC1* c.-1639G>A polymorphism changes the binding site for *VKORC1* promotor region, this results in lower *VKORC1* mRNA expression in the liver. Consequently, the concentration of tissue VKOR is lowered making a person with this variation more likely to inhibition by warfarin and also causing warfarin sensitivity (Gage *et al.*, 2008; Linder *et al.*, 2009). *VKORC1* rs9934438 (c.1173C>T) as well as rs7294 (c.3730G>A) (synonymous variant) are associated with warfarin dose variability (D'Andrea *et al.*, 2005).

1.6.3 Gamma-glutamyl carboxylase (*GGCX*)

The *GGCX* gene encodes the *GGCX* enzyme. It is located on chromosome 2p11.2 on the reverse strand (De Vilder *et al.*, 2017). The vitamin k cycles controls anticoagulation through the gamma-carboxylation of glutamic acid residues which are on the vitamin k-dependent factors (factor II, VII, IX, X, Protein S and Protein C) (Hirsh *et al.*, 2001). The carboxylation process is carried out by *GGCX* (Presnell and Stafford, 2002). *GGCX* together with vitamin k hydroquinone (K1H2) as a cofactor facilitate the carboxylation process and *VKORC1* recycles it back to K1H2 (Rieder *et al.*, 2007).

Brenner *et al.* (1998) reported mutations in *GGCX* in four patients with a deficiency in all Vitamin K Dependent Clotting Factors (VKDCF) type 1 (Brenner *et al.*, 1998). These mutations were caused by homozygous missense mutation in the gene (Brenner *et al.*, 1998; Rost *et al.*, 2004; Rost *et al.*, 2006). In addition, vitamin k cycle has an important role on haemostasis regulation and the presence of non-synonymous mutations in *VKORC1* lead to VKDCF type 2 (Rost *et al.*, 2004). Mutations in *GGCX* and *VKORC1* has drastic effects on clotting factor production, this

highlights the hypothesis that there are subtle regulatory polymorphisms in these genes that affect vitamin k cycling (Bodin *et al.*, 2005; Wang *et al.*, 2008) thereby affecting warfarin therapy.

1.7 Study rationale

Compared to European and Asian populations, African populations remain poorly studied with respect to the pharmacogenetics of warfarin. It is however known that African populations do not always possess the same genetic profiles as Caucasian or Asian populations thus the dosing algorithms that are currently available do not cater for African populations. Huge differences have been observed among African populations in dosage requirements to reach INR as well as variations in time required to reach INR.

Warfarin is extensively used among patients and may remain so for a long time to come. A study by Anakwue *et al.* (2014), reported that there are no optimum available anticoagulation service centres which provide targeted and desired outcomes in Nigeria (Anakwue *et al.*, 2014). The current available anticoagulation centres are managed by haematologists of which there are not that many. There are only two African countries that have monitoring clinics in their anticoagulation centres, namely; South Africa and Kenya (Anakwue *et al.*, 2014). The South African Anticoagulation clinics (ACCs) are managed by physicians, doctors and nurses (Jacobson *et al.*, 2009), whereas in Kenya, these clinics are managed by pharmacists (Anakwue *et al.*, 2014). Other African countries such as Nigeria which do not have adequate ACCs can adapt the South African thrombo-embolism prophylactic and treatment guidelines to assist in their plans of achieving anticoagulation practices (Jacobson *et al.*, 2009). Despite the availability of these clinics, the desired anticoagulation remains low in Africa, ranging from 7% to 30% in Kenya, which is comparable to the study by Anakwu *et al.* (2014) in Nigeria (Anakwue *et al.*, 2014). While South Africa ranges between 32% to 58% (Tiryaki *et al.*, 2011).

1.7.1 Justification

Genomics is gaining momentum in explaining some of the observed differences in drug responses, including response to warfarin. We carried out pharmacogenomics research to identify genetic markers associated with dose requirements among African populations and those of mixed ancestry origin. We were interested in investigating the contribution of genomic variation on warfarin pharmacokinetics (PK) and pharmacodynamics (PD).

The candidate PK and PD genes were *VKORC1* and *GGCX*, respectively. We had also characterised two other genes; *CYP2C9* and *CYP4F2* however, the SNPs for these genes were not validated therefore excluded from the analysis of the current study. We therefore hope that the genetic markers identified in this study may be used by clinicians to develop personalized pharmacotherapeutic regimes for their patients. This may reduce the possibility of harmful side-effects and inadequate drug responses (Arvanitidis *et al.*, 2007).

1.7.2 Study aim

The primary aim was to investigate the correlation between warfarin dose and genotypes for *VKORC1* and *GGCX*.

This was approached through these objectives:

- Identification and recruitment of patients using warfarin at INR clinics in Cape Town, South Africa
- Characterization of known candidate single nucleotide polymorphisms (SNPs) from previous literature: *VKORC1* c.-1639G>A, *VKORC1* c.1173C>T, *VKORC1* c.3730C>T, *GGCX* c.1218C>T and *GGCX* c.1242C>T
- Determination of allele and genotype frequencies of the study cohort
- Correlation of factors affecting stabilising dose

Chapter 2: Methods and Materials

2.1 Patient recruitment

2.1.1 Study site and patient recruitment

This study was conducted at the University of Cape Town, Medical School. Consenting patients were recruited from Groote Schuur Hospital (GSH) and Gugulethu Community Health Centre (GCHC) clinics. Ethical clearance was obtained from the University of Cape Town, Human Research Ethics Committee (HREC) REC REF: 581/2015. We recruited in two locations because we saw that in GSH the frequent population group visiting the clinic was the Mixed Ancestry. The patients consented to access to their demographic data and clinical information and 5 ml of blood for genetic characterisation was collected in ethylenediaminetetraacetic acid (EDTA) coated tubes.

2.1.2 Inclusion and exclusion Criteria

This study included both male and female patients above the age of 18 years who were already on warfarin therapy. The patients had any of the following conditions: atrial fibrillation (AF), deep vein thrombosis (DVT), pulmonary embolism (PE) and prosthetic/ mechanical valve replacement. A total of 372 patients were included in the genetic analysis. Patients that were excluded were those that were haemophilic as they already have a blood condition, vulnerable or not in a good mental condition, pregnant and those under 18 years.

2.2 DNA extraction and Genetic characterisation

To carry out genetic characterisation, blood was used to extract DNA which was then kept at -20°C until needed for analysis. Genomic DNA was extracted using a modified version of Gustafson et al. (1987) method (Gustafson *et al.*, 1987). Blood samples were left at room temperature to thaw before proceeding with DNA extraction. Samples were then transferred to 10 ml tubes and phosphate-buffered saline (PBS) was added up to 10 ml. The tubes were mixed by inverting the tubes and centrifuged at 2500g for 15 minutes. A reddish pellet was visible after the spin and the supernatant discarded.

The pellet was re-suspended in 10 ml of Sucrose Triton X-100 lysing buffer and vortexed before centrifugation at 2500g for 15 minutes. The supernatant was poured out carefully, with a pinkish/white pellet visible. The pinkish/white pellet was re-suspended in 3 ml T20E5, with 200 μ l 10% sodium dodecyl sulphate (SDS) and 37.5 μ l Proteinase K added, however, mixed by inversion after adding each solution. The samples were incubated in a water bath at 45°C overnight. The following day, 1 ml of saturated sodium chloride (NaCl) was added and mixed vigorously for 15 seconds then spun for 30 minutes at 2500g, a white pellet was visible which consists of protein precipitated by salt. At this point, the DNA is found in the supernatant thus the supernatant was transferred into a new tube. For precipitation of the DNA, 2 volumes of absolute alcohol were added and kept at room temperature at least 30 minutes. The samples were mixed vigorously and centrifuged for 10 minutes at 2500g and the supernatant discarded. At this point, the DNA is expected to settle at the bottom of the tube and 70% alcohol is used to wash the DNA. Samples were left to air dry for 4 hours. After 4 hours, an appropriate volume (100-250 μ l) Tris EDTA (TE) was added to dissolve the DNA and left for 3 days to dissolve the DNA at 4°C. After the 3 days, the absorbance at 260 and 280 nm was measured.

After DNA extraction, the integrity of the DNA was checked using a (w/v) 1% agarose gel (SeaKem LE Agarose, Lonza, Maine, USA) against a 100 base pair (bp) molecular marker (Thermo Fischer Scientific, Massachusetts, USA) as a reference for size. Samples were visualised using the UviTec Cambridge UV visualizer (Whitehead Scientific, MA, USA). For genetic characterisation SNPs selected were chosen based on the minor allele frequency of other African populations or African Americans.

2.2.1. Primer selection

Genetic characterisation was achieved using restriction fragment length polymorphism and Sanger sequencing method. Table 2.1 shows SNP significance and sequences of primers used. PCR conditions for the SNPs of interest are described below:

Table 2.1: SNP primers and expected fragment sizes after digestion

SNP	SNP significance	Primers	Method	PCR product size (bp)	Reference
<i>VKORC1</i> <i>c.-1639G>A</i>	Decrease RNA expression	F: GAGCCAGCAGGAGAGGGAAATAT R: GTTTGGACTACAGGTGCCTGCC	PCR/RFLP	291/ <i>Msp I</i>	(Natarajan <i>et al.</i> , 2013)
<i>VKORC1</i> <i>c.1173C>T</i>	Decrease protein expression	F: GAGCTGACCAAGGGGGA R: TGACATGGAATCCTGACGTG	PCR/RFLP	361/ <i>Hinf I</i>	(Kianmehr <i>et al.</i> , 2010)
<i>VKORC1</i> <i>c.3730G>A</i>	Decrease protein expression	F: AGCCTGATGTGGCTCAGTTT R: GTGTGGCACATTTGGTCCATT	Sanger sequencing	262	Primer design-Primer Quest Tool
<i>GGCX</i> <i>c.1218C>T</i>	Decrease protein expression	F: GGAAGGAAAGGAAACGGATGA R: CTTGAGAAAAGGCAAAGCAGAC	Sanger sequencing	381	Primer design-Primer Quest Tool
<i>GGCX</i> <i>c.1242C>T</i>	Uncertain	F: GGAAGGAAAGGAAACGGATGA R: CTTGAGAAAAGGCAAAGCAGAC	Sanger sequencing	381	Primer design-Primer Quest Tool

Note: SNP= Single nucleotide polymorphism, PCR= Polymerase chain reaction, RFLP= Restriction length polymorphism

2.2.1.1 PCR reactions and conditions

The *VKORC1* gene is located on the short arm of chromosome 16 at position 11.2. It consists of three exons (Figure 2.1) and encodes 163 amino acids. Discussed below is the PCR conditions for our SNPs of interest in the *VKORC1* gene.

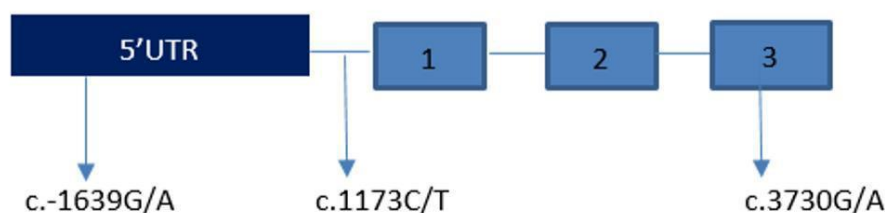


Figure 2.1: Schematic representation of the *VKORC1* gene. The positions of the *VKORC1* SNPs studied are also indicated.

VKORC1 c.-1639G>A

In a total reaction of 25 µl; 1X Green GoTaq PCR buffer (Promega, Madison, WI, USA), 3 mM of MgCl₂ (Promega, Madison, WI, USA), 0.2 mM Deoxynucleotide Triphosphates (dNTPs) (Celtic Molecular Diagnostics, Cape Town, SA), 0.32 µM of each primer (Integrated DNA Technologies, Illinois, USA), 1 unit Go-Taq (Promega, Madison, WI, USA), the reaction mix was topped up to 25 µl using sabax deionised water (Adcock Ingram, Johannesburg, SA) and 100 ng of genomic DNA was used as a template in the PCR reaction. The thermal cycling T100™ Thermal Cycler (Bio-Rad Laboratories, California, USA) was used for DNA amplification. The cycling conditions were as follows: initial denaturation at 94°C for 10 minutes, 94°C denaturation for 1 minute, 66°C annealing for 1 minute, 72°C extension for 1 minute, a total of 35 cycles were included, total extension at 72°C for 10 minutes. Samples were visualised on a (w/v) 1% agarose gel (SeaKem LE Agarose, Lonza, Maine, USA) and a 100 bp molecular marker (Thermo Fischer Scientific, Massachusetts, USA) used as a reference. 20 µl/100ml of 1X E-Z Vision gel nucleic acid stain (Thermo Fischer Scientific, Massachusetts, USA) was added for staining. PCR product size was 361 bp. The gel ran at 100 V for 45 minutes.

To digest the 291 bp amplicon, 3 µl of 10X CutSmart Buffer (New England Biolabs, Massachusetts, USA), 10 units *MspI* was added to 10 µl PCR product. The reaction was topped up to 30 µl with sabax deionised water (Adcock Ingram, Johannesburg, SA). The digestion reaction was conducted at 37°C for an hour. A 3% agarose gel was used to separate the fragments which was stained with 1X E-Z Vision gel nucleic acid stain (Thermo Fischer Scientific, Massachusetts, USA). The restriction sequence identified by *MspI* is C[^]CGG (Figure 2.2). Wild type c.-1639GG yielded two fragments 124 bp and 167 bp, the homozygous c.-1639AA yielded a single undigested 291 bp fragment and the heterozygous allele yielded 3 fragments 124 bp, 167 bp and 291 bp.

VKORC1 c.-1639G>A rs9923231 Restriction enzyme: *MSpI* (C'CGG)

Amplicon size: 291 bp

Forward primer: 5' GTTTGGACTACAGGTGCCTGCC3'

Reverse primer: 5' GAGCCAGCAGGAGAGGGAAATAT3'

Rev. complimentary: 5' ATATTTCCCTCTCCTGCTGGCTC3'

TAAGAGGTCCTTCACCAGCCTCCTCTCCCGGCATTATCCCATCTACCCCTCCACATTCAAGTTTTTGG
AAAGATTCTACACTCCCAGTCTCTACTTCTCTCACTTCTTCTTGTGCTGCCCACGCCATAAACTAGCTGC
TGCTTCCAGCATTGCCCTGACACCTAGTGGCTGGTGTACCAAGACGCTAGACCCAATGGTTATTTAT
TTATTTATTTACTTATTTTGTAGACGGAGTCTCACTCTGTGCGCCAGGCTGGAGTGCAGCGGTGCCATC
TCGGCTCACTGCAACTCCGCTCCAGGGTTCAAGTGGTTCTCGTGCCTCAGCCTCCCAAGTAGTTTG
GACTACAGGTGCCTGCCACCATGTCTGGCTAATTTTTGTATTTTGTAGTAGACAGGGTTTCACCATG
TTGGCCAGGCTTGTCTTAAACTCCTGACCTCAAGTGATCCACCCACCTCGGCCTCCCAAATGCTAGG
ATTATAGGCGTGAGCCACCGCAC(C(G/A)GGCCAATGGTTGTTTTTCAGGTCTTCTCTTGCTTGACT
TCCCAGAGGGATCCCTTACTGTTGCACCTACCTTCTGCGGAACCTCTTCTCTGCGGTCTGTGATAT
TTCCCTCTCCTGCTGGCTCCTCCCTCTCCAGATGCTGTTTCTCACATCTACTCTTCTAGAGAGTGT
GGTAGACAGAATAATGGTCACCAAGATGTCCCTGCATGAATCCCTGGAACCTGTGAATATGATAGGT
TAAATGGCCAAAAGGAATTAAGGTTGCAGATGGAATTAAGCTGACCAATCTCCTGATTTTATTTTAT
TTTATTTTGTGTTTGTAGGTGGAGTTTCGCTCTTGTGCCCCAACTGGAGTGCAATGGTGTGATCTCGGC
TCACTGCAACCTCCGCTGCCAGGTTTCGAGAGATTCTCCTGCCTCAGCCTCCCGAGTAGCTGGGATTA
CAGGCACCCGCCATCATGCCTGGCTAATTTTTTAAATTTTGTAGTAGACAGGG

Figure 2.2: Amplified region containing VKORC1 c.-1639G>A. Forward and reverse primer highlighted in green with the direction indicated by the arrows. Sequence highlighted in blue is the restriction recognition site with the red letters indicating possible variant base change which can either be a 'G' or 'A' allele.

VKORC1 c.1173C>T

In a total reaction of 25 µl; 1X Green GoTaq PCR buffer (Promega, Madison, WI, USA), 3 mM of MgCl₂ (Promega, Madison, WI, USA), 0.2 mM Deoxynucleotide Triphosphates (dNTPs) (Celtic Molecular Diagnostics, Cape Town, SA), 0.4 µM of each primer (Integrated DNA Technologies, Illinois, USA), 1 unit Go-Taq (Promega, Madison, WI, USA), the reaction mix was topped up to 25 µl using sabax deionised water (Adcock Ingram, Johannesburg, SA) and 100 ng of genomic DNA was used as a template in the PCR reaction. The thermal cycling T100™ Thermal Cycler (Bio-Rad Laboratories, California, USA) was used for DNA amplification.

The conditions for PCR cycle were as follows: initial denaturation at 94°C for 10 minutes, 94°C denaturation for 1 minute, 56°C annealing for 1 minute, 72°C extension for 1 minute, a total of 35 cycles were included, total extension at 72°C for 10 minutes. Samples were visualised on a (w/v) 1% agarose gel (SeaKem LE Agarose, Lonza, Maine, USA) and a 100 bp molecular marker (Thermo Fischer Scientific, Massachusetts, USA) used as a reference. 20 µl/100ml of 1X E-Z Vision gel nucleic acid stain (Thermo Fischer Scientific, Massachusetts, USA) was added for staining. PCR product size was 361 bp. The gel ran at 100 V for 45 minutes. To digest the 361 bp amplicon,

3 µl of 10X CutSmart Buffer (New England Biolabs, Massachusetts, USA), 10 units *HinfI* was added to 10 µl PCR product. The reaction was topped up to 30 µl with sabax deionised water (Adcock Ingram, Johannesburg, SA). The digestion reaction was conducted at 37°C for an hour. Fragments were separated on a 3% agarose gel stained with 1X E-Z Vision gel nucleic acid stain (Thermo Fischer Scientific, Massachusetts, USA). The restriction sequence identified by *HinfI* is G[^]ANTC (Figure 2.3). Wild type c.1173CC yielded two fragments 8 bp and 353 bp fragment, the homozygous c.1173TT yielded three fragments 8 bp, 43 bp and 310 bp, and the heterozygous c.1173CT yielded four fragments 8 bp, 43 bp, 310 bp and 353 bp.

***VKORC1* c.1173C>T** rs9934438 Restriction enzyme: *HinfI* (G[^]ANTC)
Amplicon size: 361 bp

Forward primer: 5'GAGCTGACCAAGGGGGA3'

Reverse primer: 5'TGACATGGAATCCTGACGTG3'

Rev. complimentary: 5'CACGTCAGGATTCCATGTCA3'

TCAAAACAACAACAACAAAAAACCCTGGGGACCTAGGATGTCCTTAAGGGCCCTTCAGCCTCTAACAG
TACTTAAACCAATTAAAAAGACTCCTGTTAGTTACCTCCCCACATCCCCACCCGAGGACGCTCCGTGA
TGAGCAGCTAGCTGGCTGTGAGCTGTGTGGATCACCAAGATTGCATGGAGTGGGGCTGAGCTGACCAA
GGGGGATGAGGGGCGGGGCGGGGCGGGCAGGGAGGGGGCGGAGCCACTCACCTAACAAATAGCTGTAGT
GTGTAGAAGATGCAACCGAATATGCTGTTGGATTGATTGAGGATGCTGTCTGTCCAGCACATGCTC
CACCAGCCCCGAAACCCCTGCCACCTGGCAGAGGGGTGGGGTGGGGTGGAAACCAGGTTAGGACTGTG
AACCAGTGCCCTGGACCCCTGCCGAGAAAGGTGATTTCCAAGAAGCCACCTGGGCTATCCTCTGTTC
CCCGACCTCCCATCCTAGTCCAAAG(C/T)GTCGATGATCTCCTGGCACCGGGCACCTTTGGC
CAGGATTCCATGTCACTGACCCTATCCTCCCTCTCCCCAGACCAGGCCCGGACGTGGCTACTCCGTA
GGCCCTGCTTTTCATCTTAGACCTTAAGTAAGTCTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGAGAC
GGAGTCTCACTCTGGCCAGGCTGGAGTGCAGTGGCGGATCTCGGCTCACTGAAACCTCCGCCTCCC
GGGTTCAAGCGATTCTCCTGCCTTAGCCCCCGAGTAGCTGGGATTAAGGCACCCGCCATAGCGCCC
GATTAATTTTCTATTTTGGTAGAGACAGGCTTTACCATGTTGGCCAGGCTGGTCAACTCCTGACT
TCAAGTGATCCATCCGCCTCGGCCTCCCAAAGTGCTGGGATTACAGGAGTGGGCCACCGGCCCGGCC
CTTAAGTAATCTTAAATGGCAAGGCTGGTATAACGGTTCACCTCGGTTTGTCA

Figure 2.3: Amplified region containing *VKORC1* c.1173C>T. Forward and reverse primer highlighted in green with the direction indicated by the arrows. Sequence highlighted in blue is the restriction recognition site with the red letters indicating possible variant base change which can either be a 'C' or 'T' allele.

***VKORC1* c.3730G>A**

In a total reaction of 25 µl; 1X Green GoTaq PCR buffer (Promega, Madison, WI, USA), 3 mM of MgCl₂ (Promega, Madison, WI, USA), 0.2 mM Deoxynucleotide Triphosphates (dNTPs) (Celtic Molecular Diagnostics, Cape Town, SA), 0.4 µM of each primer (Integrated DNA Technologies, Illinois, USA), 1 unit Go-Taq (Promega, Madison, WI, USA), the reaction mix was topped up to 25 µl using sabax deionised water (Adcock Ingram, Johannesburg, SA) and finally consisting of 100

ng/μl DNA template. The thermal cycling T100™ Thermal Cycler (Bio-Rad Laboratories, California, USA) was used for DNA amplification. Cycling conditions were as follows: initial denaturation at 94°C for 10 minutes, 94°C denaturation for 30 seconds, 58°C annealing for 30 seconds, 72°C extension for 90 seconds, a total of 35 cycles were included, total extension at 72°C for 5 minutes.

Sanger sequencing

VKORC1 c.3730G>A was genotyped using Sanger sequencing. The protocol used is as follows: firstly, the PCR products was purified from any unbound nucleotides. This was achieved by adding 1 unit FastAp thermosensitive Alkaline Phosphatase (Thermo Fischer Scientific, Massachusetts, USA), 4 units Exonuclease I (New England Biolabs®), 8.9 μl Sabax water (Adcock Ingram, Johannesburg, SA) and 15 μl in a PCR tube. The reaction mix was cleaned-up on the Thermal Cycler T100™ (Bio-Rad Laboratories, USA). The conditions were as follows: 37°C for an hour and 75°C for 15 minutes.

After the PCR clean-up was completed, we proceeded to cycle sequencing. In a total reaction of 10 μl; 0.4 μM reverse primer, 0.2X of BigDye® Terminator v3.1 dilution buffer (Life Technologies, CA, USA), 0.4X of BigDye® Terminator Sequencing Buffer (Thermo Fischer Scientific, Massachusetts, USA), 2 μl Sabax water (Adcock Ingram, Johannesburg, SA) and 3 μl cleaned-up PCR product were added. We used the GeneAmp® PCR System 9700 from Applied Biosystems (Thermo Fischer Scientific, Massachusetts, USA) to perform sequencing. Initial denaturation at 98°C for 5 minutes, for 35 cycles- denaturation at 95°C for 30 seconds, annealing at 55°C for 15 seconds and extension at 60°C for 4 minutes.

After the completion of sequencing, ethanol precipitation followed. Samples were transferred to 1.5 ml Eppendorf tube. 50 μl cold 100% ETOH and 2 μl of 3 M NaOAc pH5.2 were added to the 1.5 ml sample tubes and kept overnight at -20°C. On the following day, samples were spun at 10 000rpm for 10 minutes (Centrifuge 5415D; Eppendorf AG, Hamburg, Germany) and the supernatant removed. 35 μl of 70% ice-cold ETOH was added and sample centrifuged at 10 000rpm for 10 minutes (Centrifuge 5415D; Eppendorf AG, Hamburg, Germany). The supernatant was removed and samples air-dried for at least 4 hours.

After ethanol precipitation, capillary electrophoresis preceded. 10 µl Hi-Di formamide was added to the PCR tubes, vortexed and denatured on the thermal cycling T100™ Thermal Cycler (Bio-Rad Laboratories, USA) at 95°C for 5 minutes. Samples must be cooled down immediately in a frozen coolant for 2 minutes. Using the ABI3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), capillary electrophoresis was performed and using DNASTar® program we were able to perform gene annotation.

GGCX c.1218C>T and c.1242C>T

GGCX is located on chromosome 2p11.2 and consists of 15 exons. *GGCX c.1218C>T* and *c.1242C>T* are located on exon 9 and are 24 bp apart. Described below are the PCR conditions. In a total reaction of 25 µl; 1X Green GoTaq PCR buffer (Promega, Madison, WI, USA), 3 mM of MgCl₂ (Promega, Madison, WI, USA), 0.2 mM Deoxynucleotide Triphosphates (dNTPs) (Celtic Molecular Diagnostics, Cape Town, SA), 0.4 µM of each primer (Integrated DNA Technologies, Illinois, USA), 1 unit Go-Taq (Promega, Madison, WI, USA), the reaction mix was topped up to 25 µl using sabax deionised water (Adcock Ingram, Johannesburg, SA) and finally consisting of 100 ng/µl DNA template. The thermal cycling Thermal Cycler T100™ (Bio-Rad Laboratories, California, USA) was used for DNA amplification. The cycling conditions included initial denaturation at 94°C for 3 minutes, in a total of 35 cycles, 94°C denaturation for 30 seconds, 56°C annealing for 30 seconds, 72°C extension for 1 minute and a final extension at 72°C for 5 minutes.

Sanger sequencing

GGCX c.1218C>T and *GGCX c.1242C>T* were genotyped using Sanger sequencing. The protocol used is as follows: firstly, the PCR products was purified from any unbound nucleotides. This was achieved by adding 1 unit FastAp thermosensitive Alkaline Phosphatase (Thermo Fischer Scientific, Massachusetts, USA), 4 units Exonuclease I (New England Biolabs®), 8.9 µl Sabax water (Adcock Ingram, Johannesburg, SA) and 15 µl in a PCR tube. The reaction mix was cleaned-up on the Thermal Cycler T100™ (Bio-Rad Laboratories, USA). The conditions used were as follows: 37°C for an hour and 75°C for 15 minutes.

After the PCR clean-up was completed, we proceeded to cycle sequencing. In a total reaction of 10 µl; 0.4 µM reverse primer, 0.2X of BigDye® Terminator v3.1 dilution buffer (Life Technologies, CA, USA), 0.4X of BigDye® Terminator Sequencing Buffer (Thermo Fischer Scientific,

Massachusetts, USA), 2 µl Sabax water (Adcock Ingram, Johannesburg, SA) and 3 µl cleaned-up PCR product were added. We used the GeneAmp® PCR System 9700 from Applied Biosystems (Thermo Fischer Scientific, Massachusetts, USA) to perform sequencing. Initial denaturation at 98°C for 5 minutes, for 35 cycles- denaturation at 95°C for 30 seconds, annealing at 55°C for 15 seconds and extension at 60°C for 4 minutes.

After the completion of sequencing, ethanol precipitation followed. Samples were transferred to 1.5 ml Eppendorf tube. 50 µl cold 100% ETOH and 2 µl of 3 M NaOAc pH5.2 were added to the 1.5 ml sample tubes and kept overnight at -20°C. On the following day, samples were spun at 10 000rpm for 10 minutes (Centrifuge 5415D; Eppendorf AG, Hamburg, Germany) and the supernatant removed. 35 µl of 70% ice-cold ETOH was added and sample centrifuged at 10 000rpm for 10 minutes (Centrifuge 5415D; Eppendorf AG, Hamburg, Germany). The supernatant was removed and samples air-dried for at least 4 hours.

After ethanol precipitation, capillary electrophoresis preceded. 10 µl Hi-Di formamide was added to the PCR tubes, vortexed and denatured on the thermal cycling T100™ Thermal Cycler (Bio-Rad Laboratories, USA) at 95°C for 5 minutes. Samples must be cooled down immediately in a frozen coolant for 2 minutes. Using the ABI3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), capillary electrophoresis was performed and using DNASTAR® program we were able to perform gene annotation.

2.3 Data analysis/Statistical analysis

Descriptive statistics was performed using GraphPad Prizm version 5. Stata™ version 11 was used to identify correlation between categorical predictor and independent variables. For association between categorical variables, the Chi square (χ^2) test was performed. The χ^2 test was used to assess whether the genotype frequencies were under Hardy-Weinberg equilibrium. Weekly warfarin maintenance dose was summarised as means together with standard deviations. These means were compared among the patients considering dose requirements of different genotypes using a t-test and analysis of variance (ANOVA). Statistical significance was considered when the p value was at a level of $p < 0.05$.

Chapter 3: Results

3.1 Participant clinical and demographic features

A total of 372 South African black (SAB) and Mixed Ancestry (MA) participants were eligible for the study. There were more females on warfarin therapy than males, in both ethnic groups (Table 3.1). A statistically significant difference in age was observed between MA and SAB participants, $p < 0.0001$. There was marginal distribution of smokers between genders, 53% female and 47% male smokers. However, there were more MA smokers (62%) than SAB smokers (20%). Differences were also seen in alcohol consumption. There was a high 59% incidence for female alcohol consumers compared to the 40% observed for male alcohol consumers within the MA group. Whereas in the SAB, the incidence for male alcohol consumers was higher (52%) than the females (24%). Differences in alcohol consumption were also observed between the two ethnic groups in this study. MA had more alcohol consumers (47%) than SAB (30%), though it was not statistically significant ($p = 0.422$).

Table 3.1: Demographic and clinical information for Mixed ancestry (MA) and South African black (SAB) participants

	MA			MA total	SAB			SAB total	MA vs. SAB P value
	Males N=95	Females N=168	P value		Males N=23	Females N=86	P value		
Demographic variables									
Mean \pm SD Age	56 \pm 16,1	59 \pm 14,7	0,398	58 \pm 15,3	48 \pm 14,1	50 \pm 14,1	0,452	50 \pm 14,1	<0,0001
Mean \pm SD BMI	25 \pm 5,7	28 \pm 6,1	0,003	27 \pm 6,1	26 \pm 7,4	30 \pm 6,9	0,794	29 \pm 7,1	0,031
Tobacco smoker	72 (44%)	90 (56%)	0,616	162 (62%)	13 (56%)	9 (10%)	0,202	22 (20%)	0,495
Alcohol consumer	51 (41%)	73 (59%)	0,471	124 (47%)	12 (52%)	21 (24%)	0,303	33 (30%)	0,422
Clinical variables									
<i>Warfarin indication:</i>									
Atrial fibrillation	36 (38%)	44 (27%)	0,095	80 (31%)	7 (30%)	19 (22%)	0,27	26 (24%)	0,142
Deep vein thrombosis	11 (12%)	36 (22%)		47 (18%)	3 (13%)	20 (23%)		23 (21%)	
Pulmonary embolism	8 (8%)	18 (11%)		26 (10%)	0	9 (10%)		9 (8%)	
Mechanical valve replacement	40 (42%)	68 (41%)		108 (41%)	12 (52%)	37 (43%)		49 (45%)	
Venous thromboembolism	0	0		0	1 (4%)	1 (1%)		2 (2%)	
<i>Comorbidities:</i>									
Bleeding	14 (15%)	36 (22%)	0,191	50 (19%)	6 (26%)	19 (22%)	0,229	25 (23%)	0,202
Heart failure	31 (33%)	70 (42%)	0,384	101 (38%)	9 (39%)	24 (27%)	0,303	33 (30%)	0,36
<i>Other conditions:</i>									
Hypertension	36 (38%)	78 (47%)	0,435	114 (44%)	4 (17%)	35 (40%)	0,358	39 (36%)	0,412
Diabetes mellitus	16 (17%)	30 (18%)	0,176	46 (18%)	2 (9%)	9 (10%)	0,101	11 (10%)	0,154
HIV positive	0	7 (4%)	0,027	7 (3%)	4 (17%)	21 (24%)	0,229	25 (22%)	0,086

For warfarin indication, the most common indication in this study was mechanical valve replacement (MV), at least 40% of participants were on therapy because of MV. This is also seen between genders where 44% of male participants had MV as an indication for warfarin and 42% in females. The least frequent indication for warfarin was venous thrombosis (VT) which accounted for less than 1% of participants. Inevitably, participants were likely to experience comorbidities with warfarin therapy.

The most common comorbidity in the study was hypertension, at least 35% of participants were treated for hypertension concurrently. More males were treated for hypertension (44%) than females (36%). Looking at the common comorbidity between the ethnic groups, hypertension remained the most common comorbidity. It was higher in the MA (44%) group than SAB (36%). Diabetes was more frequent in the MA (18%) than SAB (10%). Furthermore, statistical difference ($p=0.027$) was observed in gender for HIV positive status in the MA group.

Weekly stabilising dose was correlated with participants' variables (Table 3.2). From our study cohort, age was inversely associated with stabilising dose requirements in both MA and SAB cohorts ($p<0.0001$ and $p=0.042$, respectively). The association between BMI and stabilising dose requirements was only significant in the MA cohort ($p=0.003$). Finally, in the SAB group, we observed an association between HIV positive patients and stabilising dose requirement ($p=0.020$).

Table 3.2: Univariate analysis between clinical variables and weekly stabilising dose

Variable	MA		SAB	
	Correlation coefficient (95% CI)	P-value	Correlation coefficient (95% CI)	P-value
Age	-0,275 (-0,389 to -0,160)	<0.0001	-0.102 (-0.201 to -0.036)	0.042
BMI	0.097 (0.034 to 0.159)	0.003	-0.032 (-0.087 to 0.024)	0.255
Gender	-0.003 (-0.007 to 0.0009)	0.127	0.0007 (-0.002 to 0.004)	0.635
Tobacco Smoker	0.001 (-0.003 to 0.005)	0.639	0.00005 (-0.004 to 0.002)	0.720
Alcohol consumer	0.003 (-0.001 to 0.007)	0.162	-0.001 (-0.004 to 0.002)	0.549
Warfarin Indication	0.0008 (-0.012 to 0.010)	0.888	0.007 (-0.003 to 0.017)	0.157
Bleeding events	0.0007 (-0.003 to 0.004)	0.687	0.003 (-0.0006 to 0.006)	0.116
Heart failure	-0.002 (-0.006 to 0.002)	0.384	0.0005 (-0.003 to 0.004)	0.771
Diabetes Mellitus	0.001 (-0.002 to 0.005)	0.429	-0.001 (-0.003 to 0.0008)	0.294
Hypertension	-0.002 (-0.007 to 0.002)	0.274	-0.0005 (-0.004 to 0.003)	0.784
HIV positive	-0.0001 (-0.0015 to 0.0013)	0.884	0.004 (0.0006 to 0.007)	0.020

3.2 Genetic analysis

3.2.1 Sample preparation and genetic characterization

3.2.1.1 DNA extraction and quality control

DNA extraction was successful in 372 participants. Quality control was conducted on the blood samples after DNA extraction, using a modified salting out method adapted from (Gustafson *et al.*, 1987), by running a 1% integrity gel (Figure 3.1) to assess if the DNA was intact or degraded.

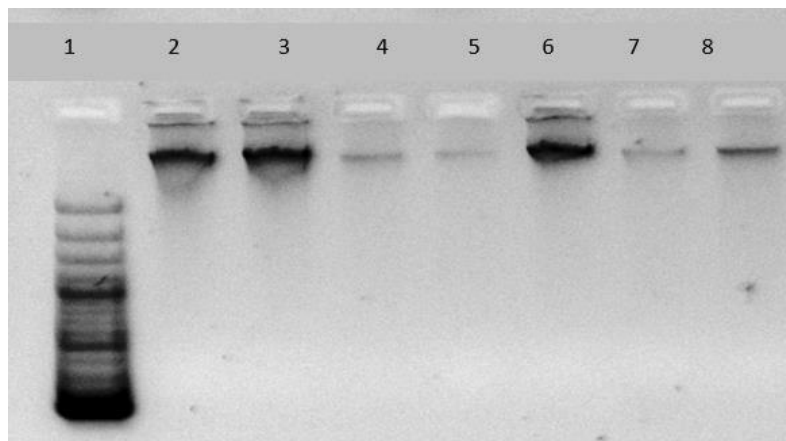


Figure 3.1: 1% Integrity gel indicating an example of samples of SAB and MA participants. Lane 1 is the 100 bp (Thermo Fisher Scientific GeneRuler, Wilmington, DE, USA), molecular weight marker. Lane 2-8 samples showing the presence of DNA after DNA extraction.

3.2.1.2 PCR-RFLP Gel Electrophoreses and Sanger sequencing results

For *VKORC1* c.-1639G>A genotype determination, PCR-RFLP method was used and 372 samples were successfully amplified. The wild-type G/G presented two bands: 124 bp and 167 bp. Heterozygotes, G/A, had three bands: 124 bp, 167 bp and 291 bp. The mutant genotype, A/A, was the undigested 291 bp band. Genotypes were validated using Sanger sequencing as seen on Figure 3.2.

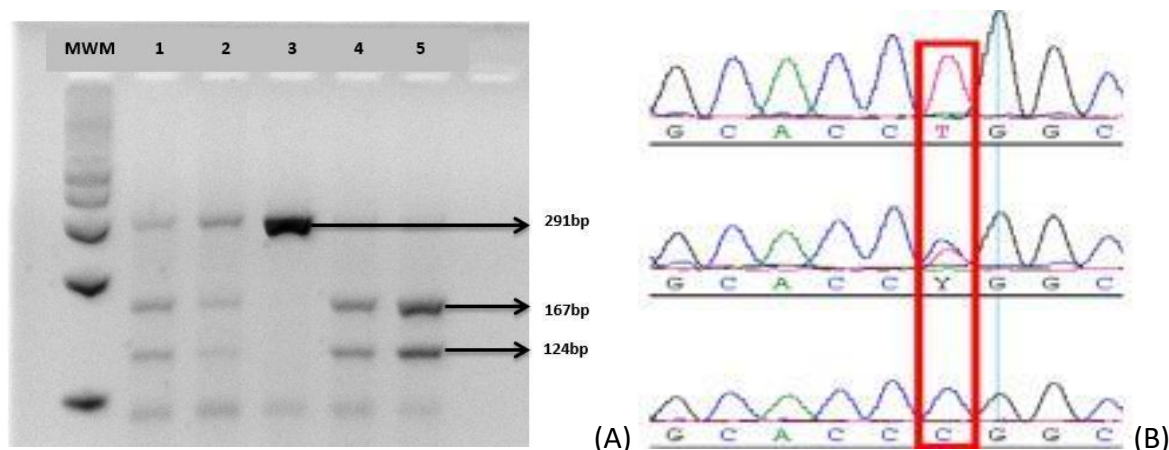


Figure 3.2: *VKORC1* c.-1639G>A PCR – RFLP Gel Electropherogram (A) and Sanger Sequence chromatograph (B).

Figure (A) represents a 3% agarose gel showing results of *VKORC1* c.-1639G>A PCR amplicon digested with *MspI* restriction enzyme. Lane 1: 100 bp (Thermo Fisher Scientific GeneRuler, Wilmington, DE, USA) molecular weight marker. Lane 4 and 5: Wildtype (G/G). Lane 1 and 2: Heterozygous (G/A). Lane 3: Homozygous mutant (A/A). Validation of genotypes was performed using Sanger sequencing as seen on Figure (B). The red box indicates the SNP position with different peaks for the possible bases.

VKORC1 c.1173C>T genotype characterisation was determined by PCR-RFLP method was used and 372 samples were successfully amplified. The wild-type genotype C/C was identified by the 353 bp band. The heterozygotes, C/T, were identified by two bands: 353 bp and 310 bp, the 43 bp band could not be visualised. The homozygous mutant T/T was identified by the 310 bp band and 43 bp which could not be visualised. Using the 100 bp molecular weight marker (Figure 3.3), all bands were the expected sizes.

For *VKORC1* c.3730G>A genotyping, Sanger sequencing, method was used. Using the reverse primer, clear peaks (Figure 3.4) were visible to identify genotypes. Heterozygotes were represented by the letter 'R' on the chromatogram.

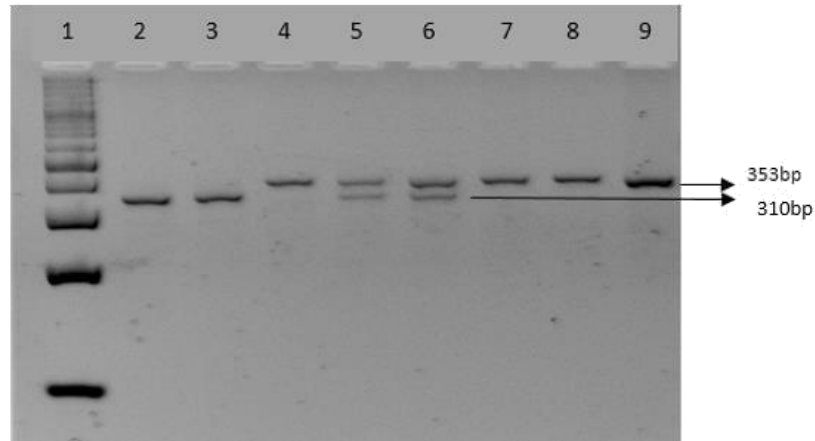


Figure 3.3: *VKORC1* c.1173C>T PCR – RFLP Gel Electropherogram. A 3% agarose gel showing results for *VKORC1* c.1173C>T PCR amplicon digested with *Hinf I* restriction enzyme. Lane 1: 100 bp GeneRuler, (Thermo Fisher Scientific Wilmington, DE, USA) molecular weight marker. Lane 2 and 3: Homozygous mutant (T/T). Lane 4, 7, 8 and 9: Wildtype (C/C). Lane 5 and 6: Heterozygous (C/T). The 43 bp fragment not visible.

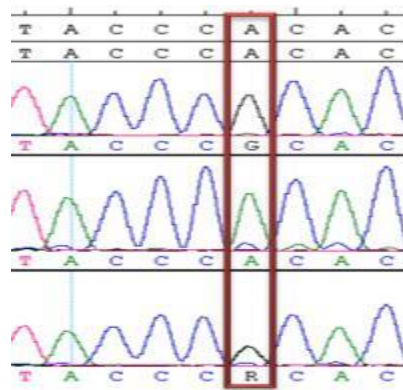


Figure 3.4: *VKORC1* c.3730G>A chromatograph. After Sanger sequencing, chromatograph indicates the variation of peaks which represent different genotypes for this SNP.

In the *GGCX* gene, we identified two SNPs which were 24bp apart; *GGCX* c.1218C>T and *GGCX* c.1242C>T, respectively. The frequency of *GGCX* c.1218T was 0.179 in SAB and 0.134 in MA. For *GGCX* c.1242T the frequency was 0.087 in SAB and 0.122 in MA. Figure 3.5 shows a chromatograph highlighting the two *GGCX* SNPs. Heterozygotes were represented by the letter 'Y'.

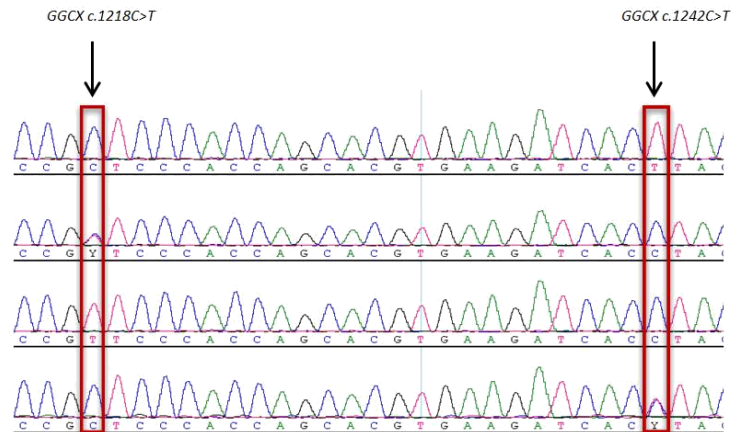


Figure 3.5: *GGCX* c.1218C>T and *GGCX* c.1242C>T chromatograph. Clear peak variations indicating different nucleotide bases.

3.2.2 Genotype and allele frequencies

After PCR-RFLP and Sanger sequencing, participant genotype frequencies were determined (Table 3.3). The chi square test was used to validate if genotypes are in HWE. In the SAB, *VKORC1* c.1173C>T and *GGCX* c.1218C>T were the only SNPs in Hardy Weinberg equilibrium (HWE); whereas in the MA, *GGCX* c.1218C>T was the only SNP not in HWE. The SNPs that were not in HWE suggests that there was possibly a type 1 error (genotyping error), this is seen with p values < 0.05 or this was due to samples not being randomised because we only selected patients on warfarin therapy.

Table 3.3: Observed genotype frequencies distribution of MA and SAB and patients

SNP	Genotype	SAB			MA		
			Genotype frequency	P-value		Genotype frequency	P-value
<i>*VKORC1</i> <i>c.-1639G>A</i>	G/G	90	0.83	0.046	130	0.5	0.52
	G/A	16	0.15		106	0.4	
	A/A	3	0.03		26	0.1	
<i>*VKORC1</i> <i>c.1173C>T</i>	C/C	80	0.73	0.39	141	0.54	0.1
	C/T	28	0.26		95	0.36	
	T/T	1	0.01		26	0.1	
<i>*VKORC1</i> <i>c.3730G>A</i>	G/G	42	0.39	0.004	73	0.28	0.791
	G/A	39	0.36		127	0.48	
	A/A	28	0.26		59	0.23	
<i>*GGCX</i> <i>c.1218C>T</i>	C/C	48	0.59	0.43	127	0.58	0.02
	C/T	27	0.33		70	0.32	
	T/T	6	0.07		21	0.1	
<i>*GGCX</i> <i>c.1242C>T</i>	C/C	65	0.8	0.043	160	0.73	0.481
	C/T	13	0.16		52	0.24	
	T/T	3	0.04		6	0.03	

Note=* N=109 for SAB and N=263 for MA. Samples did not always add up to these totals due to errors that occurred with SNP characterization.

We further determined the minor allele frequency and compared it with other populations to see if there are any similarities in the distribution of the frequencies (Table 3.4). The distribution of the mutant alleles varies across populations. Higher frequencies of the *VKORC1* variants were observed in the MA group compared to SAB, for example, in *VKORC1 c.-1639A* (0.302 in MA vs. 0.101 in SAB). Differences were observed with the *GGCX* variants. For *GGCX c.1218T*, a higher frequency is observed in SAB compared to MA (0.179 and 0.134, respectively). While *GGCX c.1248T* had a higher frequency in MA than SAB (0.122 vs. 0.087, respectively).

Table 3.4: Allele frequency table representing frequencies of different populations compared with our study cohort

Minor allele frequency							
SNP	Variant allele	SAB N=109	MA N=263	Yoruba N=113	Caucasian N=113	Asian N=43	Afr American N=347
<i>VKORC1 c.-1639G>A</i>	A	0,101	0,302	0,025	0,387	0,941	0,125
<i>VKORC1 c.1173C>T</i>	T	0,138	0,281	0,022	0,398	0,941	0,13
<i>VKORC1 c.3730G>A</i>	A	0,257	0,468	0,535	0,362	0,058	0,402
<i>GGCX c.1218C>T</i>	T	0,179	0,134	0,406	0,335	0,349	0,222
<i>GGCX c.1242C>T</i>	T	0,087	0,122	0,009	0,121	0,035	0,098

Note: N=number of participants; Afr American=African American; SNP=single nucleotide polymorphism

Surprisingly, the SAB and Yoruba populations had different frequencies though they are of African descent we would expect similar observations. *VKORC1 c.-1639A* is more frequent in Asian populations (0.941) compared to populations of African descent such as Yoruba (0.025). Similarly, Asian populations have a high frequency of *VKORC1 c.1173T* (0.941) than African descent populations, for instance, Yoruba (0.022). *VKORC1 c.3730A* was more prevalent in African descent populations, for instance, Yoruba (0.535), MA (0.468) and less common in Asian populations (0.058).

GGCX c.1218T is comparable between Asians and Caucasians (0.349 and 0.335, respectively) however, differences are observed in our South African cohort (0.179 in SAB and 0.134 in MA). For *GGCX c.1242T*, higher frequency is observed in the MA group compared to other populations like Yoruba where it is prevalent at a lower frequency (0.009).

3.3 Identified SNPs and their relationship with stabilising warfarin weekly dose requirements

Weekly stabilising dose requirements for different genotypes were determined (Table 3.5). We observed that the SAB participants required higher warfarin doses across all genotypes relative to MA participants. This could be the result of cultural differences that affect diet and lifestyle

habits. We observed a higher incidence of HIV positive patients within the SAB group (22% vs. 3%) with the HIV treatment potentially affecting warfarin response resulting in the higher warfarin dose requirements. Further investigation should assess the role of HIV treatment and warfarin response. Statistical difference observed in dose requirements in the MA cohort for *VKORC1* c.-1639G>A, *VKORC1* c.1173C>T and *VKORC1* c.3730G>A ($p<0.0001$, $p<0.0001$ and $p=0.002$, respectively). Given the statistical difference observed for these genotypes, if we assess the daily dose requirements for one of these genotypes (i.e *VKORC1* c.1173T/T) it averages to 3.6 mg/day, this is lower than the current prescribed dose at Groote Schuur Hospital or Gugulethu Community Health Centre of 5 mg/day. This indicates the need for population specific algorithms. There was no statistical difference in dose requirements in the SAB cohort for our SNPs of interest, a larger sample size could possibly increase statistical power. *VKORC1* c.-1639A and *VKORC1* c.1173T are associated with warfarin sensitivity resulting in lower warfarin dose requirements. However, in SAB for these variants, higher doses were observed. This is because for *VKORC1* c.1639A/A, the mean dosage was obtained from one out of the three individuals due to missing data in patient folders. For *VKORC1* c.1173T/T, there was only one individual with this genotype and we therefore cannot conclude on the findings because a larger number of individuals could give a better result.

Table 3.5: Weekly stabilising dose requirements for SNP genotypes

SNP	A/O	Average stabilising dose in mg/week \pm SD (N)							
		Mixed Ancestry				South African Black			
		AA	AO	OO	P-value	AA	AO	OO	P-value
<i>VKORC1</i> c.-1639G>A	G/A	38 \pm 14 (130)	31 \pm 15 (106)	20 \pm 14 (26)	<0.00 01	46 \pm 31 (90)	41 \pm 14 (16)	73 \pm 0 (3)	0.289
<i>VKORC1</i> c.1173C>T	C/T	38 \pm 15 (141)	29 \pm 14 (95)	25 \pm 13 (26)	<0.00 01	47 \pm 31 (80)	41 \pm 15 (28)	50 \pm 0 (1)	0.757
<i>VKORC1</i> c.3730G>A	G/A	29 \pm 15 (73)	34 \pm 15 (127)	38 \pm 16 (59)	0.002	42 \pm 13 (42)	45 \pm 28 (39)	54 \pm 44 (28)	0.558
<i>GGCX</i> c.1218C>T	C/T	31 \pm 15 (127)	36 \pm 16 (70)	35 \pm 15 (21)	0.175	42 \pm 16 (48)	47 \pm 32 (27)	38 \pm 6 (6)	0.949
<i>GGCX</i> c.1242C>T	C/T	34 \pm 16 (160)	31 \pm 14 (52)	31 \pm 16 (6)	0.527	44 \pm 23 (65)	43 \pm 14 (13)	43 \pm 22 (3)	0.558

Note: SNP=single nucleotide polymorphism, N=number of participants genotyped for each SNP, A=alternate allele, and O= variant allele

Genetic variants were correlated with stabilising weekly warfarin dose (Table 3.6). In the SAB participants, there was no association of the SNPs with weekly stabilising dose requirements. However, in MA participants, again the *VKORC1* SNPs showed statistical association with dose requirements. *VKORC1* c.-1639G>A and *VKORC1* c.1173C>T were inversely associated with weekly stabilising dose requirements indicating warfarin sensitivity thus lower dose requirements. In addition, *VKORC1* c.3730G>A was associated with higher weekly stabilising dose requirements in the same group.

Table 3.6: Correlation of weekly stabilising dose requirements with SNPs

SNP	MA (N=263)		SAB (N=109)	
	Correlation coefficient (95% CI)	P-value	Correlation coefficient (95% CI)	P-value
<i>VKORC1</i> c.-1639G>A	-0.0137 (-0.019 to -0.008)	<0.0001	-0.0003 (-0.004 to 0.003)	0.839
<i>VKORC1</i> c.1173C>T	-0.0138 (-0.0192 to -0.008)	<0.0001	-0.001 (-0.004 to 0.002)	0.550
<i>VKORC1</i> c.3730G>A	0.0103 (0.004 to 0.016)	<0.001	0.004 (-0.002 to 0.0102)	0.147
<i>GGCX</i> c.1218C>T	0.005 (-0.0001 to 0.116)	0.102	0.001 (-0.006 to 0.008)	0.755
<i>GGCX</i> c.1242C>T	-0.003 (-0.007 to 0.002)	0.247	-0.004 (-0.009 to 0.002)	0.218

N=number of participants genotyped for each SNP, SNP=single nucleotide polymorphism

A multivariate analysis was conducted for the SNPs with weekly stabilising dose requirements (Table 3.7) for MA participants. *VKORC1* c.-1639G>A was inversely associated with weekly stabilising dose requirements (p=0.003). The mutant genotype required a lower weekly warfarin stabilising dose (Figure 3.6), than the current prescribed dose (35 mg/week) in Groote Schuur Hospital and Gugulethu Community Health Centre. For the MA participants, the *VKORC1* c.-1639G>A SNP proved to be an important SNP which should be considered when initiating genotype-guided dosing. With our SNPs of interest, there was no association observed with SAB and weekly warfarin stabilising dose requirements. This suggests that these SNPs were not significant in dose requirements for SAB participants, therefore, other SNPs should be investigated that will assist in genotype-guided dosing for SAB.

Table 3.7: Multivariate analysis of weekly stabilising dose requirements and SNPs

SNP	Correlation coefficient (95% CI)	P-value
<i>VKORC1</i> c.-1639G>A	-7.5464 (-12.4494 to -2.6435)	0.003
<i>VKORC1</i> c.1173C>T	-0.5645 (-5.3652 to 4.2363)	0.817
<i>VKORC1</i> c.3730G>A	1.3626 (-1.9070 to 4.6323)	0.412
<i>GGCX</i> c.1218C>T	1.6990 (-1.40242 to 4.8005)	0.563
<i>GGCX</i> c.1242C>T	-1.2379(-5.4506 to 2.9749)	0.563

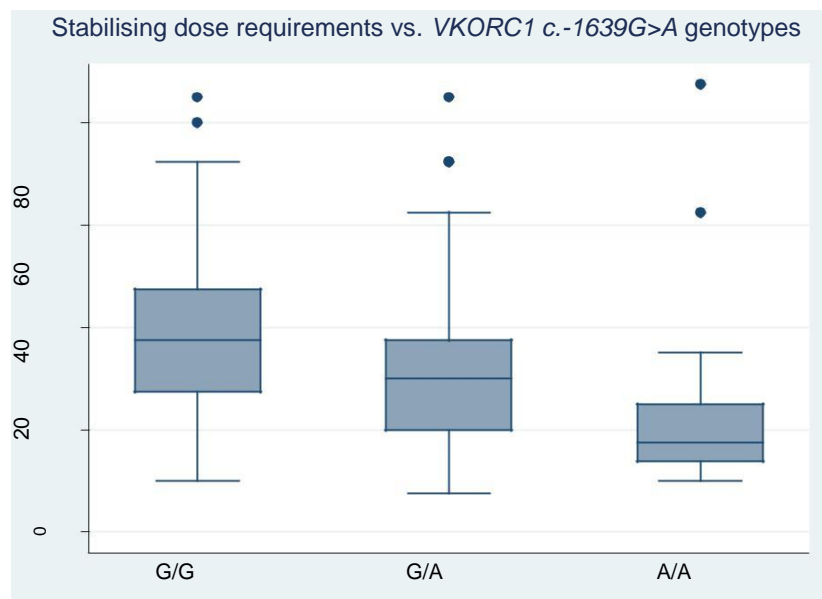


Figure 3.6: Graphic representation of weekly stabilising dose requirements in MA. Association of weekly stabilising warfarin dose requirements for *VKORC1* c.-1639G>A. A/A genotype participants required a lower dose than G/G genotype patients.

Chapter 4: Discussion

Genomic markers associated with dose requirements among African populations and those of mixed ancestry origin in the Western Cape Province, South Africa, could possibly explain the differences in drug response including warfarin response. With observation we recruited participants from Groote Schuur Hospital (n=407) and Gugulethu Community Health Centre (n=46). With the disproportion of patients in GSH, we applied at the Western Cape Government for approval to recruit patients in GCHC to increase the number of SAB patients. Recruitment in GSH was quicker than in GCHC. This is because (1) the clinic is open Monday to Thursday (2) at least 20 patients are seen on each day (3) in GSH, 407 patients were recruited and in GCHC, 47 patients were recruited and (5) the clinic in GCHC is open only on Mondays with few patients seen on that day. Duration of recruitment in GSH was from October 2015 to November 2015 while in GCHC recruitment was only in June 2016. A total of 81 patients were excluded from genetic analysis for the following reasons: (1) Unsuccessful DNA extraction resulting in the loss of DNA due to DNA extraction protocol not adhered to, (2) Pharmacogenetics of Caucasian and Asian populations has been extensively studied therefore these patients were not included in this study and (3) Patients younger than 18 years. The participants self-identified their ancestry as either black African or Mixed Ancestry from the identity of their families through 3 generations. The final sample size was 372 participants of which 263 were of Mixed Ancestry (MA) descent and 109 South African Black (SAB) participants. The participants were mostly recruited from Groote Schuur Hospital located in the Cape metropolitan area.

The Cape metropolitan is a rapidly growing low-to-middle-income area of South Africa. The rapid growth has also increased the burden of cardiovascular disease (CVD) with risk factors including smoking, high cholesterol, obesity, diabetes, high blood pressure and physical inactivity (Smith, Ralston and Taubert, 2012). In addition, this upsurge has been attributed to progressive urbanization which has promoted unhealthy lifestyles (Press *et al.*, 2010). Accordingly, the major indication for warfarin in this study was mechanical valve replacement (> 40%) likely due to heart conditions as expected in sub-Saharan Africa (Cappuccio and Miller, 2016). While warfarin remains the drug of choice in this area. Interestingly, in South African studies by Schapkaitz and Sithole. (2017) and Sonuga *et al.* (2016), the common indication for warfarin indication was atrial fibrillation (36.1% and 65%, respectively) (Sonuga *et al.*, 2016; Schapkaitz and Sithole, 2017).

Similar results were observed in a Chinese study by Liu et al. (2017), atrial fibrillation was the most common indication (33.3%) (Liu *et al.*, 2017). Nielsen et al. (2017) also reported on atrial fibrillation as the most common indication (54%) in a Danish study by (Nielsen *et al.*, 2017).

The primary goal of warfarin therapy is to maintain a stable International normalised ratio (INR) with the lowest effective dose possible (Kuruvilla and Gurk-Turner, 2001; Pirmohamed, 2013). For this study, weekly stabilising warfarin dose requirements was inversely associated with age in the MA and SAB groups ($p < 0.0001$ and $p = 0.042$, respectively). Similar findings were observed in a study by Khoury and Sheikh-Taha. (2014), where warfarin maintenance dose was lower in older patients (Khoury and Sheikh-Taha, 2014). These findings correspond with published literature, warfarin dose requirements decrease with increasing age (Merli, 2005; Singla and Morrill, 2005; Whitley *et al.*, 2007). This is as a result of decreased metabolic activities, serum proteins and renal excretion (Crooks *et al.*, 1976) in older patients. Consequently, the persistence of the drug in the body is increased by the changes in the drug pharmacokinetics resulting in increased drug sensitivity among older patients (Miura *et al.*, 2009).

For this study, BMI had an influence on weekly stabilising warfarin dose requirements in the MA group ($p = 0.003$) with a weak correlation $r = 0.246$. similar findings were reported in an African-American population study, Whitley et al (2007), where a weak correlation ($r = 0.08$) was observed between Total Weekly warfarin Dose (TWD) and BMI (Whitley *et al.*, 2007). On the other hand, BMI had no influence on weekly stabilising warfarin dose in the SAB group. This was consistent with previously reported studies that found no relationship between BMI and warfarin dose requirements (Oates *et al.*, 1998; Blann *et al.*, 1999). These contradicting results makes it challenging to draw on a firm conclusion on the effects of BMI and warfarin stabilising dose. Conflicting results are also observed when assessing the effects of warfarin stabilising dose and variables such as tobacco smoking, alcohol consumption and warfarin indication.

Firstly, tobacco smoking had no effect ($p > 0.6$) on stabilising warfarin dose. In a systemic review by Nathisuwan et al. (2011) conflicting evidence on the effects of smoking on warfarin dosage was gathered (Nathisuwan *et al.*, 2011). In their investigation, some studies suggested a significant 12% increase in warfarin dose requirements among smokers (Millican *et al.*, 2007; Gage *et al.*, 2008; Lenzini *et al.*, 2008). This interaction with warfarin may increase warfarin clearance and reduce its effect (Lucas and Martin, 2013), thereby promoting dose increase.

Other studies found no association with smoking (Lee *et al.*, 2005; Aquilante *et al.*, 2006; Whitley *et al.*, 2007).

Secondly, alcohol consumption had no effect ($p>0.1$), however, Roth *et al.* (2012) reported an association between alcohol and major bleeding risk ($p=0.04$) (Roth *et al.*, 2015). Weathermon and Crabb. (1999) suggest that alcohol should be avoided completely during warfarin therapy (Weathermon and Crabb, 1999). This is because alcohol has the likelihood to increase warfarin anticoagulation effects which will increase the INR, thereby increasing the risk of haemorrhage (Ilaris *et al.*, 2009). However, if alcohol is consumed within normal limits it is safe (Ilaris *et al.*, 2009). It is therefore important to educate patients to avoid excessive use of alcohol (Sonuga *et al.*, 2016).

Finally, we did not have any association of warfarin indication with stabilising warfarin dose for this study. However, Anderson *et al.* (2004) found lower warfarin dose requirements for patients who suffered from stroke and this is a result of undernutrition which is expected after stroke (Andersson *et al.*, 2004). In addition, Limdi *et al.* (2009) found that patients with severe chronic kidney disease required significantly low warfarin dose ($p=0.002$) (Limdi *et al.*, 2009). Moreover, Lenzini *et al.* (2010) found that lower warfarin requirements were recommended when using diabetes as a marker (Lenzini *et al.*, 2010) and this was consistent with previously published literature (Hillman *et al.*, 2004). In contrast, comorbidities had no effect on warfarin dosing in this study.

Warfarin remains the drug of choice for anticoagulant treatment. The metabolism of warfarin is affected by genetic polymorphisms in cytochrome P450 2C9 (*CYP2C9*) and vitamin k reductase complex 1 (*VKORC1*) both of which account for 30%-40% of warfarin dose variation (Bodin *et al.*, 2005; Sconce *et al.*, 2005; Vecsler *et al.*, 2006). Candidate genes that have been identified include gamma-glutamyl carboxylase (*GGCX*), epoxide hydrolase 1 (*EPHX1*), calumenin (*CALU*) and *CYP2C19* (Wadelius *et al.*, 2007; Luxembourg *et al.*, 2010; Huang *et al.*, 2011). Some of these genes were not screened for because of time constraints.

4.1 Correlation between genetic variation and warfarin stabilising doses

In the present study, we focused on SNPs found in two genes (*VKORC1* and *GGCX*). Firstly, in the *VKORC1* gene, the allele frequency for *VKORC1* c.-1639A was observed at 10% in SAB. Geisen *et al.* (2005) observed similar findings in an African American study with a frequency of 14% (Geisen

et al., 2005). In addition, an Egyptian study by Ghozlan *et al.* (2005) also observed a frequency of 10% (Ghozlan *et al.*, 2015). These similar findings are expected because these populations are of African descent and therefore may have a common ancestor. In the MA, *VKORC1* c.-1639A frequency was 30%, similar to observations in a US study by Budnitz *et al.* (2007) where they observed a frequency of 29.7% (Budnitz *et al.*, 2007). However, higher frequencies (84%) were observed in an Asian population (Li *et al.*, 2015). From the *VKORC1* c.-1639G>A genotypes, the highest mean weekly stabilising warfarin dosages in this study was given to carriers of the A/A genotype, while carriers of the heterozygous genotype G/A were treated with lower mean weekly stabilising warfarin dosages (73 ± 0 mg/week and 41 ± 14 mg/week, respectively) in SAB. Dosages from only one individual of the A/A genotype were available. However, in the MA group, there was significant mean weekly dose requirements for the *VKORC1* c.-1639G>A ($p < 0.0001$). The highest mean weekly stabilising warfarin dosages was given to carriers of the G/G genotype (wild-type), while carriers of the mutant genotype A/A were treated with lower dosages (38 ± 14 mg/week and 18 ± 14 mg/week, respectively) which is equivalent to (5.4 ± 2 mg/day and 2.6 ± 2 mg/day, respectively). In a study by Natarajan *et al.* (2013), carriers of the G/G genotype received higher daily doses than A/A genotype carriers (4.7 mg/day and 2 mg/day, respectively) (Natarajan *et al.*, 2013). The higher dose requirement for the G/G was also seen in this study.

Secondly, for *VKORC1* c.1173T, a 14% allele frequency was observed in SAB and 28% in MA. Mclellan *et al.* (2011) observed a 4% frequency in a South African black population (Mclellan *et al.*, 2011). In addition, Takahashi *et al.* (2006) observed a frequency of 8% in African Americans which was lower than observations in Caucasians and Asians (42% and 89%, respectively) (Takahashi *et al.*, 2006). For *VKORC1* c.1173C>T, the carriers of the T/T genotype in SAB required higher mean stabilising warfarin dose and lower mean stabilising warfarin dosages were administered to C/T genotype (73 ± 0 mg/week and 41 ± 14 mg/week, respectively), this is because there was only one individual with the T/T genotype in SAB. In comparison, in the MA group, significant mean dose requirements for the *VKORC1* c.1173C>T were observed ($p < 0.0001$). The C/C genotype received higher mean weekly stabilising warfarin dosages than the T/T genotype (38 ± 15 mg/week and 25 ± 13 mg/week, respectively). Different findings were observed by Kianmehr *et al.* (2010) in an Iranian population where the C/C and T/T genotype each received weekly warfarin dose of 26.4 ± 15.8 mg/week and 15 ± 6.2 mg/week, respectively (Kianmehr *et al.*, 2010). A negative association was observed for *VKORC1* c.-1639G>A and *VKORC1* c.1173C>T and weekly stabilising warfarin dose in SAB. While a significant negative association was observed for *VKORC1* c.-1639G>A and *VKORC1* c.1173C>T in the MA group

($p < 0.0001$) with mean weekly stabilising warfarin dose indicating low dose phenotypes. Similarly, Geisen et al. (2005) found comparable results (Geisen *et al.*, 2005). On the other hand, for *VKORC1* c.3730G>A, a positive association was observed in SAB and a significant positive association was observed in MA ($P < 0.001$) with weekly stabilising warfarin dose requirements. Kringen et al. (2011) observed similar findings where *VKORC1* c.3730G>A was associated with high warfarin dose phenotype (Kringen *et al.*, 2011).

Lastly, for *VKORC1* c.3730A the SAB the allele frequency was 26% and 47% in MA. Interestingly, similar findings to the MA, a South African black population frequency (40%) was observed (McLellan *et al.*, 2011). Limdi et al. (2008) observed frequencies of 20.3% in African Americans while Takahashi et al. (2006) observed a frequency of 52% (Takahashi *et al.*, 2006; Limdi *et al.*, 2008). It is interesting to note the differences in frequency distribution among these populations of African descent is likely due to differences in sample size. For *VKORC1* c.3730G>A polymorphism which is associated with warfarin resistance, carriers of the A/A genotype were administered higher mean weekly warfarin doses than G/G carriers (wild-type) (54 ± 44 mg/week and 42 ± 13 mg/week, respectively) in SAB. In MA, A/A carriers received higher mean weekly warfarin dosages than the wild-type G/G genotype (38 ± 16 mg/week and 29 ± 15 mg/week, respectively). Cini et al. (2012) had different observations for mean weekly dose requirements for A/A and G/G genotypes (56.7 ± 13.3 mg/week and 25.3 ± 8.8 mg/week, respectively) (Cini *et al.*, 2012) but they also agree to the warfarin resistance association with this SNP.

The *GGCX* gene has not received much attention. To our knowledge, this is the first study on *GGCX* variation and its correlation with warfarin among African populations. For this gene, we were interested in the 1218C>T and 1242C>T SNPs. For the *GGCX* c.1218T variant, the frequency was 18% in SAB and 13% in MA. In an Asian population study by Kamali et al. (2013), they observed a frequency of 28% (Kamali *et al.*, 2013). In addition, Shikata et al. (2004) in a Japanese population observed a frequency of 68% (Shikata *et al.*, 2004). For the *GGCX* c.1242T we observed a frequency of 8% in SAB and 12% in MA. The observation in SAB was similar to a Japanese study by Shikata et al. (2004) where they observed a frequency of 6% (Shikata *et al.*, 2004). These SNPs are not commonly studied thus, limited literature is available. The probable reason for this is that the clinical effect of these SNPs is benign. When looking at the *GGCX* gene; for *GGCX* 1218C>T in SAB, though not statistically significant, there were differences in mean weekly warfarin dosage administration observed in heterozygous C/T genotype and T/T genotype

(47 ± 32 mg/week and 38 ± 6 mg/week, respectively). In the MA group, heterozygous C/T genotype received higher mean warfarin dosages than C/C genotype (36 ± 16 mg/week and 31 ± 15 mg/week, respectively). These findings were different than observations made by Kamali et al. (2010) in an Asian population. They found that the T/T genotype required higher doses than the C/C genotype (28.4 ± 6.2 mg/week and 20 ± 4.3 mg/week, respectively) (Kamali and Wynne, 2010). For *GGCX* c.1242C>T, carriers of the C/C genotype received higher doses than C/T genotypes (44 ± 23 mg/week and 43 ± 23 mg/week, respectively) in SAB. Similarly, in the MA group, carriers of C/C genotype received higher doses than C/T genotype (34 ± 16 mg/week and 31 ± 14 mg/week, respectively). Furthermore, for the *GGCX* c.1242C>T SNP, we observed a negative association with weekly stabilising dose in both groups ($r=-0.004$ in SAB and $r=-0.003$ in MA). More genetic analysis should be done on this SNP among diverse populations to find the effect it has on stabilising dose.

With multivariate analysis, we assessed the effect of our SNPs of interest on weekly stabilising dose requirements in the MA group since significant association was observed in some of the SNPs. *VKORC1* c.-1639G>A maintained significant negative association with stabilising dose requirements. This emphasised the warfarin low dose phenotype. As a result, *VKORC1* c.-1639G>A genotype testing is required for warfarin dosing in the MA group.

4.2 Limitations

We had a small sample size for the SAB ($n=109$) compared to the MA ($n=273$). This small sample size may not have been enough to extrapolate the findings on warfarin dose treatment. This limitation was a result of failure in DNA extraction for this group. In addition, some participants had missing data for their clinical records leading to valuable information not captured.

Conclusion

From this study, we determined allelic variants of *VKORC1* and *GGCX* in a South African population. To our knowledge, variants alleles of *GGCX* have not been reported in an African population. The *GGCX* variant alleles can be used in future studies particularly of African descent to investigate any correlation with warfarin response.

There was no genetic association for warfarin dose requirements with our SNPs of interest in the SAB group. However, we observed an association ($p=0.033$) of dose requirements in the MA group for *VKORC1* c.-1639G>A SNP. Carriers of the G/G genotype had higher dose requirements (38 ± 14 mg/week) compared to carriers of the A/A genotype (18 ± 14 mg/week).

From our observations, environmental factors such as age and BMI influenced warfarin dose requirements in this population. Age was inversely associated with weekly stabilising dose requirements ($p>0.001$ in MA; $p>0.042$ in SAB), with increasing age there is a lower dose requirement. In addition, it was only in the MA group that an association with weekly stabilising dose and BMI was observed ($p=0.003$).

We conclude that both genetic and environmental factors may influence warfarin response in South African patients. Differences observed between MA and SAB population groups are likely due to underlying genetic markers for warfarin response. Therefore, functional studies may assist in the determination of the effect of these variants in cellular models. In addition, knowledge in the pharmacogenomics of warfarin can help improve warfarin therapy among South African patients. To achieve this, more genetic markers need to be characterized as it may assist in improved warfarin therapy.

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Appendix I

INFORMATION LEAFLET AND CONSENT FORM

STUDY TITLE: PHARMACOGENOMICS OF COUMARIN DRUGS: A FOCUS ON THE GENETIC DETERMINANTS AFFECTING WARFARIN RESPONSE

PRINCIPAL INVESTIGATORS: Professor Collet Dandara and Professor Mpiko Ntsekhe

OTHER STAFF INVOLVED: Zinhle Cindi, Edson Makambwa, Dr Miguel Larceda and Dr Lerato Mpye

INSTITUTION: University of Cape Town, Division of Human Genetics

Introduction

Hello, I am _____*, a researcher in the Division of Human Genetics, UCT. I am approaching you because you are on treatment with a medication called Warfarin and I would appreciate it if you were to participate in this research project. I would be very grateful for your participation. Before agreeing to participate, it is important that you understand the following explanations of the purpose of the study, the study procedures, benefits, risks, discomforts, and precautions as well as your right to withdraw from the study at any time. This information leaflet is to help you to decide if you would like to participate. You should fully understand what is involved before you agree to participate.

Purpose of the study

This study is being undertaken in order to find out what causes differences in the time it takes for patients on warfarin to reach a stable dose that is safe. We are asking whether patients getting different times to reach this safe dose also have different changes in their genes. Genes are the molecules in our bodies that tells us how to function. They are contained in DNA which is what each of us inherits from our parents. This DNA can be extracted from blood and can be used to tell us if there are any changes on the DNA that may be associated with the differences that we observe in drug response. If you decide to participate, you will not be asked to take any additional drugs except what your doctors have prescribed for your condition.

Procedures

If you agree to participate, your medical information including demographic information will be accessed from the clinic files and you will be required to complete a questionnaire, one asking for information on your ethnicity and clinical records.

A qualified nurse will draw 5 ml blood from you for the extraction of DNA that will be used for genetic analysis. Possible side effects which may be associated with obtaining a blood sample include pain, bruising, light-headedness and on rare occasions infection. Precautions will be taken to avoid these difficulties. The entire procedure should take approximately 10 minutes.

Sample collection schedule

After consenting to participate in the study, the following schedule will be followed for collection of study samples from you.

Activity	Duration	Follow up activity
Interview	10-15min	Blood collection

Unforeseen risks and potential benefits

Regular medication will continue however, should you experience side effects please contact your doctor immediately. Your participation in the study does not have direct benefit, however, these findings may assist dosing for future patients.

As a participant in the study you have the following rights: Your participation is completely voluntary and free. Should you participate, you are still free to withdraw from the study and there is no need to give reasons for your withdrawal. This will have no effect on the standard of care that you receive or your relationship with the doctor. The test results will be made available to you on your request. The results will be strictly confidential and published data will not have your name.

Some of the methods used for DNA characterization have a potential to identify other genetic mutations that are known to be associated with other disease risks.

Ethical approval: *This clinical study protocol has been approved by the University of Cape Town, Human Research Ethics Committee (HREC) REC REF: 581/2015.*

Source of additional information: Your prescribing doctor is still facilitating your care throughout the duration of the study whom you should contact at any time should you feel that any of your symptoms are causing you problems. Should you have questions relating to the study please contact Professor Dandara, on the following 021 406 6506 or 0849955010 and email: collet.dandara@uct.ac.za

If you agree to participate, please sign the consent form. Thank you.

Appendix II

Consent form

Participant name: _____ Participant number: _____

I have read or heard the explanation of this study and I am willing to participate.

Please indicate ☒ your consent for the following:

Storage and analysis of my DNA for the warfarin study Yes ☐ /No ☐

Access of my medical records for the warfarin study Yes ☐ /No ☐

Notification of my doctor of any medically-actionable incidental findings Yes ☐ /No ☐

Participant's signature

Date

Witness signature

Date

Researcher's signature

Date

Appendix III

VOLUNTEER QUESTIONNAIRE

PROJECT TITLE: PHARMACOGENOMICS OF COUMARIN DRUGS: A FOCUS ON THE GENETIC DETERMINANTS AFFECTING WARFARIN RESPONSE

Participant name: _____ Participant number: _____

We need information about you, your parents and your grandparents in order to determine if there is any genetic difference in the metabolism of drugs between different Southern African populations. Please try to be as accurate as possible. The quality of data will depend on the truth of your response to the questions below. It is better to leave out information than to give us something that might be wrong.

1. ABOUT YOURSELF

Gender (sex): _____ Date of birth: (yyyy/mm/dd) _____

Place of birth: _____ Province/country: _____

Home language: _____ other-language: _____

Contact address: _____

Tel/Cell: _____

2. ABOUT YOUR PARENTS

Your mother

Your father

Place of birth: _____

Province or country: _____

Home language: _____

3. ABOUT YOUR GRANDPARENTS

Your mother's mother

Your father's mother

Place of birth: _____

Province or country: _____

Home language: _____

Your mother's father

Your father's father

Place of birth: _____

Province or country: _____

Home language: _____

Appendix IV

PHARMAGENOMICS OF WARFARIN QUESTIONNAIRE

Participant name: _____ Participant number: _____

Please complete the following demographic information questionnaire and take note that your answers to the following questions will be used for research purposes only and will be kept strictly confidential.

Date of birth (yyyy/mm/dd)					
Gender	Male		Female		
Ethnicity <input type="checkbox"/> (Please tick where applicable)					
Afrikaner	English	Mixed ancestry	Indian	Sipedi	
Sotho	Tswana	Venda	Swazi	Ndebele	
Tsonga	Xhosa	Asian	Other (specify)		
Height		Weight			
Do you smoke tobacco (tick ✓)					
• Yes	How many cigarettes per day do you or did you smoke				
• In the past					
• No	If you have stopped, when was that?				
Do you consume alcohol (tick✓)?					
• Yes	How much alcohol per day or per week?				
• In the past	When did you start (month/year)				
• No	If you have stopped, when was that?				
What type of alcohol (tick✓)?					
	• Lager				
	• Wine				
	• Spirits				
	• Home-made				
	• Cider				
Level of Qualification <input type="checkbox"/> (Please tick where applicable)					
	None	Primary	High School	Matric	Tertiary
Employment <input type="checkbox"/>					
	Still studying	Employed	Unemployed	Retired	
Type of employment					
Conditions indicated for: <input checked="" type="checkbox"/> (Please tick where applicable)	Atrial fibrillation	Venous thromboembolism	Prosthetic/mechanical valves	Deep vein thrombosis	Pulmonary embolism
Initiation date for treatment (yyyy/mm/dd)					
Number of warfarin dose adjustment before reaching INR (mg/d)					
	2 nd dose	3 rd dose	4 th dose	More	
Date when INR was achieved (2 weeks within target)					
	Date	Dose INR (mg/d)			
Any bleeding events/ adverse reactions on warfarin					
Yes	No	Date & description:			
Concomitant drugs use (Name and duration)					
Other comorbidities e.g. blood pressure, diabetes etc.					
Yes	No	Comments:			
Other parameters					
Adverse drug event					
Date/comments					
Diet (any special diet, frequency of eating green vegetables, fruits)					

Appendix IV

Western Cape study approval



STRATEGY & HEALTH SUPPORT

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5th Floor, Norton Rose House, 8 Riebeeck Street, Cape Town, 8001
www.capegateway.gov.za

REFERENCE: WC_2016RP34_557
ENQUIRIES: Ms Charlene Roderick

University of Cape Town
Anzio Road
Observatory
7935

For attention: **Prof Collet Dandara**

Re: **PHARMACOGENOMICS OF COUMARIN DRUGS: A FOCUS ON THE GENETIC DETERMINANTS AFFECTING WARFARIN RESPONSE.**

Thank you for submitting your proposal to undertake the above-mentioned study. We are pleased to inform you that the department has granted you approval for your research. Please contact the following people to assist you with any further enquiries in accessing the following sites:

Gugulethu CHC

Lunga Makamba

Contact No: 021 637 1280

Kindly ensure that the following are adhered to:

1. Arrangements can be made with managers, providing that normal activities at requested facilities are not interrupted.
2. Researchers, in accessing provincial health facilities, are expressing consent to provide the department with an electronic copy of the final feedback (**annexure 9**) within six months of completion of research. This can be submitted to the provincial Research Co-ordinator (Health.Research@westerncape.gov.za).
3. In the event where the research project goes beyond the *estimated completion date* which was submitted, researchers are expected to complete and submit a progress report (**Annexure 8**) to the provincial Research Co-ordinator (Health.Research@westerncape.gov.za).
4. The reference number above should be quoted in all future correspondence.

Yours sincerely


DR. A. HAWKRIDGE
DIRECTOR: HEALTH IMPACT ASSESSMENT
DATE: 21/04/2016
CC: FOLCKERS

DIRECTOR: KLIPFONTEIN/ MITCHELLS PLAIN

Appendix V

DNA extraction protocol

PURIFICATION OF DNA FROM FROZEN BLOOD (for 5 ml sample) Collet Dandara (modified from Gustafson et al., 1987)

1. Thaw blood at room temperature or in a room temp water bath.
2. Transfer to a sterile polypropylene tube, rinse with 1ml PBS
3. **Dilute with 2 volume of PBS** (1 mM KH_2PO_4 , 154 mM NaCl, 5.6 mM Na_2HPO_4 , pH 7.4)
4. Mix by inverting the tube
5. Centrifuge at 2200 g for 10 min
6. Carefully pour off the supernatant. The pellet will be reddish.
7. Resuspend the pellet in 10 mL of **Sucrose Triton X-100 Lysing Buffer** & vortex
8. Place on ice for 5 minutes
9. Spin for 7 minutes or longer at 2200g (2300rpm in **TH.4** rotor)
10. Pour off the supernatant **carefully**. The pellet should now be pinkish or white.
11. Resuspend the pellet in 3 mL of **T20E5** (0.6X volume of original blood) 3 ml
Add **10% SDS** to a final concentration of 1 % 200ul
Add **Protenase K**, 10 mg/ML to a final concentration of 250 ug/mL ~~100ul~~
37.5
Mix by inversion after adding each solution
12. Incubate the samples at 45°C >2hours, preferably overnight.
13. Add 1mL of saturated NaCl and mix vigorously for 15 seconds
14. Spin for 10 minutes at 2400g. A white pellet should be visible which consists of protein precipitated by salt
15. Transfer the supernatant that contains the DNA to a new tube
16. In order to precipitate the DNA, add 2 volumes (approx. 9ml) of absolute alcohol kept at room temperature.
17. Invert 50 times gently, place in freezer for 20 minutes.
18. Spin for 10 minutes at 2400g. Mark pellet and remove supernatant.
19. Wash in 70% ice-cold alcohol (add 1mL)
20. Spin for 10 minutes at 2400g. Remove supernatant.
21. Air dry the DNA or dry at 42 degrees
22. Dissolve the DNA in the appropriate volume of **TE** (50-100uL)

Appendix VI

VKORC1 c.-1639G>A PCR-RFLP Protocol

1. PCR

Reagents	Volume (μL) Single reaction	Final Concentration
Template DNA (100 ng/μl)	1	
5X GoTaq Flexi PCR Buffer (Promega)	5	1X
25 mM GoTaq MgCl ₂ (Fermentas)	3	3 mM
5 mM dNTPs	1	0.2 mM
10 μM VKORC1 c.-1639G>A Forward Primer	0.8	0.32 μM
10 μM VKORC1 c.-1639G>A Reverse Primer	0.8	0.32 μM
5 U/μL Go Taq	0.1	1 unit
Nuclease free water	13.3	
Total reaction volume	25	

1.2.1 Run the PCR on the BioRadT100 Thermocycler using the following conditions:

- Initial denaturation: 94°C for 10 minutes
- Denaturation: 94°C for 1 minute
- Annealing: 65°C for 1 minute
- Extension: 72°C for 1 minute **Repeat steps ii to iii 35 times**
- Final extension: 72°C for 10 minutes

1.2.2 Prepare a 1% agarose gel and add 20 μL/100ml 1X E-Z vision gel stain. Use the 100 bp (Thermo Fischer Scientific, Massachusetts, USA) ladder as your molecular weight marker. Run the gel for 90 minutes at 100 V. The desired PCR product is 291 bp.

2. Digestion

2.1 Prepare the digestion mix as follows:

Reagents	Volume (μ L) Single reaction	Final Concentration
PCR Product	10	
10 U/ μ L MsP1(Biolabs)	1	1 unit
10X Cut Smart Buffer (Biolabs)	3	1X
Nuclease free water	16	
Total reaction volume	30	

2.2 Run the digest in the water-bath at 37°C for 3 hours.

2.3 Run the digests on a 3% agarose gel at 100 V for 2 hours.

2.4 The expected genotype RFLPs are:

Genotype	Fragment 167 bp	Fragment 124 bp	Fragment 291 bp
Homozygous G/G			
Heterozygous G/A	✓	✓	✓
Homozygous A/A			✓

Appendix VII

VKORC1 c.1173C>T PCR-RFLP

1. PCR

Reagents	Volume (µL) Single reaction	Final Concentration
Template DNA (100 ng/µl)	1	
5X GoTaq Flexi PCR Buffer (Promega)	5	1X
25 mM GoTaq MgCl ₂ (Fermentas)	3	3 mM
5 mM dNTPs	1	0.2 mM
10 µM VKORC1 c.1173C>T Forward Primer	0.5	0.4 µM
10 µM VKORC1 c.1173C>T Reverse Primer	0.5	0.4 µM
5 U/µL Go Taq	0.1	1unit
Nuclease free water	13.9	
Total reaction volume	25	

2.2.1 Run the PCR on the BioRadT100 Thermocycler using the following conditions:

- Initial denaturation: 94°C for 10 minutes
- Denaturation: 94°C for 1 minute
- Annealing: 56°C for 1 minute
- Extension: 72°C for 1 minute ***Repeat steps ii to iii 35 times***
- Final extension: 72°C for 10 minutes

2.2.2 Prepare a 1% agarose gel and add 20 µl/100ml of 1X E-Z Vision gel nucleic acid stain). Use the 100 bp molecular marker (Thermo Fischer Scientific, Massachusetts, USA). Run the gel for 90 minutes at 100 V. The desired PCR product is 361 bp.

2. Digestion

2.1 Prepare the digestion mix as follows:

Reagents	Volume (μL) Single reaction	Final Concentration
PCR Product	10	
10 000 U/ml BsrBI (Biolabs)	1	1 unit
10X Cut Smart Buffer (Biolabs)	3	1X
Nuclease free water	16	
Total reaction volume	30	

2.2 Run the digest in the water-bath at 37°C for 3 hours.

2.3 Run the digests on a 3% agarose gel at 100 V for 90 minutes.

2.4 The expected genotype RFLPs are:

Genotype	Fragment 8 bp	Fragment 43 bp	Fragment 310 bp	Fragment 353 bp
Homozygous C/C	✓			✓
Heterozygous C/T	✓	✓	✓	✓
Homozygous T/T	✓	✓	✓	

Appendix VII

VKORC1 c.3730G>A PCR Protocol

1. PCR

Reagents	Volume (μL) Single reaction	Final Concentration
Template DNA (100 ng/μl)	1	
5X GoTaq Flexi PCR Buffer (Promega)	5	1X
25 mM GoTaq MgCl ₂ (Fermentas)	2.5	3 mM
5 mM dNTPs	0.5	0.2 mM
10 μM VKORC1 c.3730G>A Forward Primer	0.5	0.4 μM
10 μM VKORC1 c.3730G>A Reverse Primer	0.5	0.4 μM
5 U/μL Go Taq	0.1	1 unit
Nuclease free water	14.9	
Total reaction volume	25	

2.3 Run the PCR on the BioRadT100 Thermocycler using the following conditions:

- Initial denaturation: 94°C for 10 minutes
- Denaturation: 94°C for 30 seconds
- Annealing: 58°C for 30 seconds
- Extension: 72°C for 90 seconds ***Repeat steps ii to iii 35 times***
- Final extension: 72°C for 5 minutes

2.4 Prepare a 1% agarose gel. Use 100 bp the (Thermo Fischer Scientific, Massachusetts, USA ThermoFisher) ladder as your molecular weight marker. Run the gel for 1 hour at 100 V. The desired PCR product is 262 bp.

Appendix IX

GGCX c.1218C>T and GGCXc.1242C>T PCR Protocol

Reagents	Volume (μL) Single reaction	Final concentration
Template DNA (100 ng/μl)	1	
5X GoTaq Flexi PCR Buffer (Promega)	5	1X
25 mM GoTaq MgCl ₂ (Fermentas)	3	3 mM
5 mM dNTPs	1	0.2 mM
10 μM Forward Primer	1	0.4 μM
10 μM Reverse Primer	1	0.4 μM
5 U/μL Go Taq	0.1	1 unit
Nuclease free water	12.9	
Total reaction volume	25	

Run the PCR on the BloRadT100 Thermocycler using the following conditions:

- Initial denaturation: 94°C for 3 minutes
- Denaturation: 94°C for 30 seconds
- Annealing: 56°C for 30 seconds
- Extension: 72°C for 1 minute ***Repeat steps ii to iii 35 times***
- Final extension: 72°C for 5 minutes

Prepare a 1% agarose gel. Use 100 bp the (Thermo Fischer Scientific, Massachusetts, USA ThermoFisher) ladder as your molecular weight marker. Run the gel for 1 hour at 100 V. The desired PCR product is 381 bp.

Appendix X

Sequencing Protocol

1. Preparation of post PCR clean up mix:

Reagents	Volume (μL) Single reaction	Final concentration
FastAp thermosensitive Alkaline Phosphatase (1 U/μL)	1	1 unit
Exonuclease I (20 U/μL)	0.2	4 units
Nuclease free water	13.8	
PCR Product	5	
Total reaction volume	20	

2. Perform the clean up using these conditions on the BioRad MyCycler Thermal Cycler:
 - i. 37°C for 1 hour
 - ii. 75°C for 15 minutes
3. Using a 3% agarose gel, check the quality of the clean-up described
4. Preparation of the sequencing mix (**NOTE: master mix should contain one primer, either the forward or reverse primer**)

Reagents	Volume (μL) Single reaction	Final concentration
10 μM Primer	1.0	0.4 μM
2.5X Big Dye Terminator mix	2.0	0.2X
5X Big Dye Sequencing Buffer	2.0	0.4X
Nuclease free water	3.5	
Clean-up PCR Product	1.5	
Total reaction volume	10.0	

1. Using the ABI9700 Thermal Cycler, perform the sequencing reaction using these conditions:
 - a. 98°C for 5 minutes
 - b. 96 °C for 30 seconds
 - c. 55°C for 15 seconds
 - d. 60 °C for 4 minutes**Repeat steps b and c for 35 cycles**
2. For a 10 μL sequencing reaction add 50 μL 100% ethanol into each tube and 2 μL sodium acetate (pH 5.2).
3. Add sample to tubes, vortex and leave overnight at -20°C.
4. Spin the samples at 10 000rpm for 10 minutes.
5. Remove supernatant.

6. Add 35 μ L of 70% ice cold ethanol to each tube, vortex and spin at 10 000rpm for 10 minutes.
7. Remove supernatant and leave to air dry for at least 4 hours.
8. Add 10 μ l of Hi-Di formamide once samples have dried.
9. At 95°C denature the samples for 5 minutes.
10. Cool the samples for 2 minutes in a frozen coolant.
11. Proceed to capillary electrophoresis using the ABI3130xl Genetic Analyzer.
12. Perform gene annotation using DNASTar® program.